UNIVERSIDAD AUTÓNOMA DE MADRID
DEPARTAMENTO DE BIOQUÍMICA

DE LA BÚSQUEDA DE LA FUNCIÓN DE LA PROTEÍNA Vmp1 A LA CARACTERIZACIÓN DE LA AUTOFAGIA EN *Dictyostelium discoideum*

TESIS DOCTORAL

JAVIER CALVO GARRIDO
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A mis padres y a mi hermano, con el mayor del cariño y admiración.

“Ninguna célula sabe quien eres ni le importa”

Daniel Dennett

“El aleteo de una mariposa en Arizona puede generar una tormenta en Hawai”

Teoría del caos

"Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad.

Albert Einstein

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RESUMEN

Dictyostelium discoideum es un microorganismo eucariota simple que presenta una serie de características biológicas que le hacen ser un modelo óptimo para el estudio de la proteína de función desconocida Vmp1 ("Vacuole membrane protein 1"). La ausencia de homólogos de esta proteína en levaduras modelo como *Saccharomyces cerevisiae* y *Schizosaccharomyces pombe* hacen de *Dictyostelium* el modelo experimental más sencillo donde caracterizar su función.

Los estudios de localización subcelular han mostrado que Vmp1 es una proteína transmembrana localizada en el retículo endoplásmico y parcialmente en autofagosomas en *Dictyostelium discoideum*.

La cepa deficiente en *vmp1* muestra una serie de defectos fenotípicos en procesos celulares tales como la macropinocitosis, la fagocitosis, la secreción proteica convencional, la autofagia, así como un severo defecto en la fase de desarrollo multicelular. La biogénesis de orgánulos como la vacuola contráctil, el retículo endoplásmico y el aparato de Golgi también se encuentra impedida.

El estudio pormenorizado de los defectos en autofagia de la cepa deficiente en *vmp1* mostró la formación de agregados proteicos ubiquitinados que colocalizaban con el marcador autofágico GFP-Atg8 y la ausencia de autofagosomas maduros. Encontramos además que diversos procesos dependientes de autofagia como la supervivencia al ayuno y la capacidad de formación de células tallo *in vitro* se encontraban impedidos.

Podríamos pensar que la función de Vmp1 es común a los procesos que derivan del retículo endoplásmico y que están relacionados con el tráfico de membranas, como la formación de autofagosomas, de la vacuola contráctil, o de las vesículas de macropinocitosis y fagocitosis. Debemos también considerar la posibilidad de que un defecto severo en autofagia pueda ser el causante del resto de fenotipos.

La autofagia está regulada a nivel molecular por la acción concertada de diversas proteínas agrupadas en complejos funcionales que actúan en diferentes estadios de la formación de los autofagosomas, son las proteínas Atg ("autophagy-related proteins").

Dictyostelium posee homólogos de casi todas ellas y además cabe señalar la presencia de algunas de ellas conservadas entre *Dictyostelium* y mamíferos y que como Vmp1 no se encuentran el levaduras.

Hemos caracterizado en *Dictyostelium* la posible presencia de agregados proteicos ubiquitinados en las cepas mutantes de los genes autofágicos *atg1*, *atg5*, *atg6*, *atg7* y *atg8*. Algunas de estas cepas, al igual que la deficiente en *vmp1*, muestran agregados proteicos ubiquitinados. Estos poseen un tamaño que correlaciona de manera positiva con la severidad del fenotipo que muestran en procesos como el crecimiento en condiciones axénicas, supervivencia en ayuno o desarrollo. Las cepas mutantes de *vmp1* y *atg1* poseían agregados de mayor tamaño que las de *atg5* y *atg7* mientras que las cepas mutantes de *atg6* y *atg8* ya no mostraban agregados. Estos resultados sugieren una clara relación entre autofagia y formación de agregados proteicos en este sistema, al igual que ocurre en diversas enfermedades neurodegenerativas.

SUMMARY

Dictyostelium discoideum is a simple eukaryotic microorganism, with several biological characteristics which make it an excellent model to address the study of the protein of unknown function Vmp1 (Vacuole membrane protein 1). Furthermore, Vmp1 is absent in yeast models such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, thus making *Dictyostelium discoideum* the simplest model to characterize Vmp1 function.

Subcellular localization studies in *Dictyostelium discoideum* have shown that Vmp1 is a transmembrane protein localized in the endoplasmic reticulum and partially in autophagosomes.

A *vmp1* mutant strain shows a variety of phenotypic defects in cellular processes such as macropinocytosis, phagocytosis, conventional protein secretion, contractile vacuole biogenesis, endoplasmic reticulum, Golgi apparatus, autophagy as well as a severe defect in multicellular development.

A detailed study of the autophagic defects in the *vmp1* mutant strain has revealed the presence of ubiquitinated protein aggregates, which colocalize with the autophagy marker GFP-Atg8, and also a lack of autophagosomes. In addition, a range of autophagy related processes such as survival to starvation and *in vitro* stalk cell differentation were found impaired in these *vmp1* mutant cells.

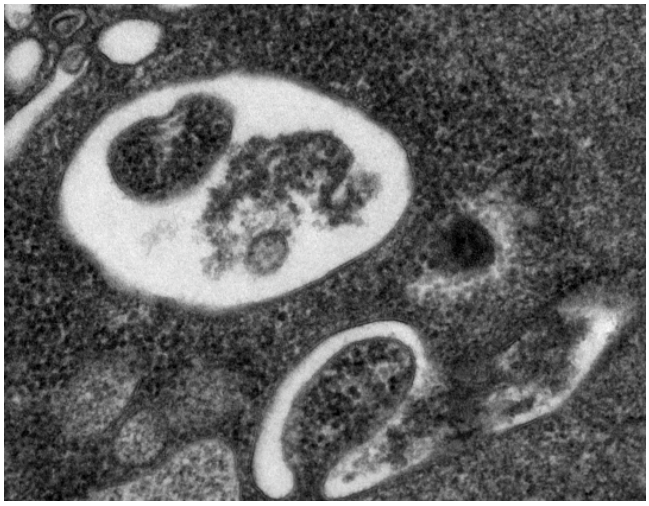
We might assume then, that Vmp1 function is common to processes arising from the endoplasmic reticulum and thus, it could be related with membrane traffic such as the autophagosomes, contractile vacuole or macropinocytosis and phagocytosis vesicles. We should also consider the possibility that the severe defect in autophagy could be the main cause of the rest of the phenotypes.

Autophagy is regulated at the molecular level by several protein complexes (Atg proteins or autophagy-related proteins) which work at different levels in autophagosome formation. *Dictyostelium* possesses homologues for most of this Atg proteins, being some of them conserved between *Dictyostelium* and mammals and absent in yeast, like Vmp1.

Summary

We have characterized *Dictyostelium* mutant strains *atg1*, *atg5*, *atg6*, *atg7* and *atg8* ubiquitinated protein aggregates. Just like in *vmp1* null some of this strains show ubiquitinated protein aggregates. The size of the aggregates correlates with the severity of the phenotype of these strains in axenic growth, survival to starvation or development. *Vmp1* and *atg1* mutant strains show bigger aggregates and a more severe phenotype than *atg5* and *atg7* mutant strains, which in turn show a more severe phenotype than *atg6* and *atg8* mutant strains which do not have such aggregates.

These results suggest a clear linkage between autophagy and protein aggregate formation in *Dictyostelium* as observed in various neurodegenerative diseases.



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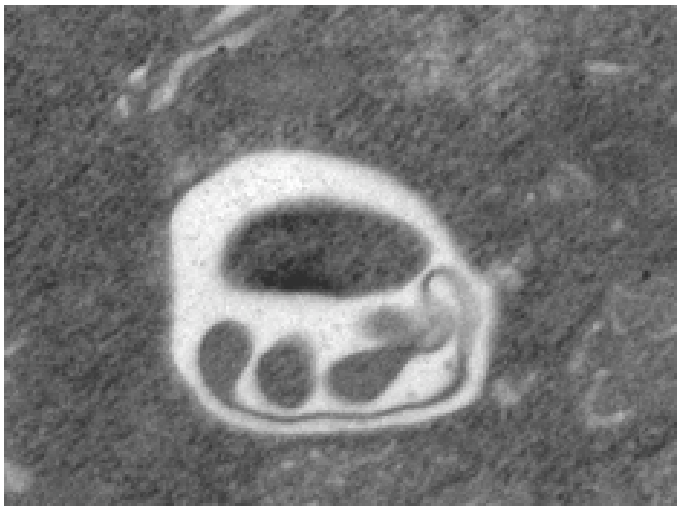

ABREVIATURAS

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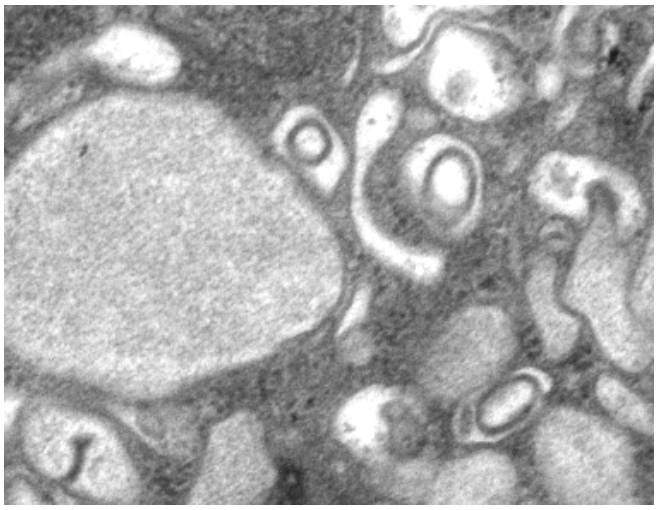
AMC	Autofagia mediada por chaperona.
Atg	Autophagy related protein.
AVP	Ácido valproico.
cAR	cAMP receptor.
CMF	Conditioned medium factor.
Cvt	Cytoplasm-to-vacuole targeting.
DIF-1	Differentiation inducing factor.
Gbf	G-box binding factor.
GFP	Green fluorescent protein.
Hsc70	Heat shock cognate 70 protein.
InsP3	Inositol 1,4,5-triphosphate.
K.O.	Knock out.
Lamp2a	Lysosomal-associated membrane protein 2a.
LC3	Microtubule-associated protein 1A/1B light chaín 3.
PDI	Protein disulfide isomerase.
PIP3	Phosphatidylinositol-3,4,5-triphosphate.
PKA	cAMP-dependent protein kinase.
PSF	Pre-starvation factor.
REMI	Restriction enzyme mediated integration.
S-1-P	Sphingosine-1-phosphate.
S-1-P-liasa	Sphingosine-1-p-liasa.
SDF1	Spore differentiation factor 1.
SDF2	Spore differentiation factor 2.
TAP-TAG	Tandem affinity purification.
TORC1	Target of rapamycin complex I.
UVRAG	UV resistance-associated gen.
Vmp1	Vacuole membrana protein 1.
ZO-1	Zonula occludens 1.



INTRODUCCIÓN

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Dictyostelium discoideum

Descubrimiento, vida y filogenia de *Dictyostelium discoideum*

Fue Oskar Brefeld en 1869 quien encontró inicialmente el primer dictiostélido, se trataba de *Dictyostelium mucoroides*, sin embargo fue otra especie diferente, *Dictyostelium discoideum,* la que comenzó a usarse como organismo modelo.
Dictyostelium discoideum es un microorganismo eucariota con un tamaño de 10μm usado en laboratorios científicos desde 1935 (Brefeld 1869). El nombre de *Dictyostelium* tiene su origen en el griego: "dictio" que significa red y "stelium" que significa torre.

Dictyostelium discoideum, también llamado ameba social, puede colonizar diferentes habitats, desde suelos húmedos de bosques protegidos del viento y sol, donde fueron encontrados inicialmente, hasta muchos otros más secos, donde se alimenta de bacterias y levaduras por fagocitosis (Cavender 1976, Cavender 1978, Romeralo et al. 2010a).

En presencia de alimentos, *Dictyostelium discoideum* es un organismo unicelular, sin embargo ante la ausencia de nutrientes se activa un complejo y regulado programa de expresión génica que deriva en la formación de un organismo pluricelular compuesto de un tallo que soporta las esporas (Escalante and Vicente 2000).

La posición filogenética de *Dictyostelium* ha sido fuente de debate desde el siglo XIX hasta la actualidad, encontrándose en continuo cambio y discusión. Inicialmente de Bary, solamente mediante criterios morfológicos los consideró muy cercanos a los protozoos, sin embargo, posteriormente, debido a que muchos de esos estudios fueron realizados por micológos, fueron clasificados dentro del reino hongos (Raper 1935). Actualmente la llegada de nuevas técnicas de biología molecular supuso un avance al aportar un nuevo enfoque al análisis de la situación filogenética de los dictiostelidos (Romeralo, Spiegel and Baldauf 2010b). El estudio de la secuencia de genes como *ssu*, *α-tubulina* o el *factor de elongación 1-α* (Baldauf and Doolittle 1997)

clasificaron a dictiostélidos (*Dictyostelium discoideum*) junto con protostélidos (*Planoprotostelium*) y mixomicetes (*Physarum*) constituyendo un grupo monofilético llamado Mycetozoa (Loomis and Smith 1995), estando más cercanos filogenéticamente dictiostélidos y mixomicetes entre sí que dictiostélidos y protostélidos.

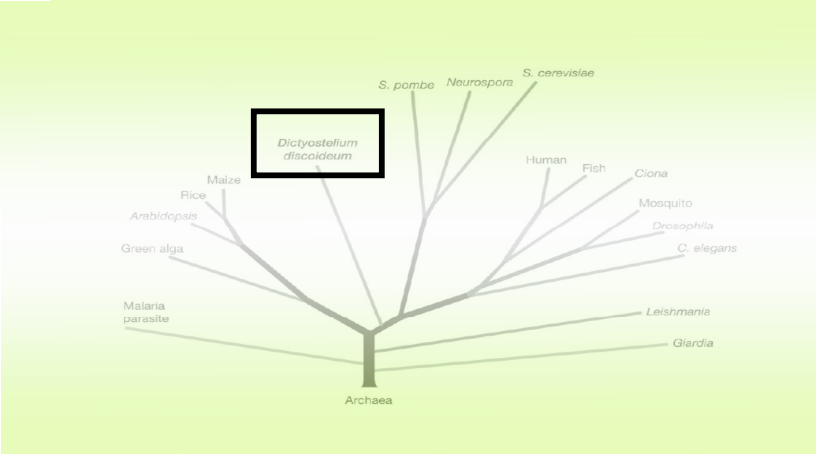


Fig.1. Arbol filogenético que muestra la posición de *Dictyostelium discoideum*. *Dictyostelium* diverge del reino animal después de que lo hagan las plantas y antes que hongos y levaduras. Los estudios morfológicos y genéticos no han conseguidos encuadrar a *Dictyostelium discoideum* en ninguno de los reinos existentes.

Características genómicas

Dictyostelium discoideum es un organismo haploide cuyo genoma consta de 34 Mb divididas en 6 cromosomas y unos 12500 genes, estando compuesto el 87% del ADN por adeninas y timinas. Posee pocos intrones, siendo el tamaño de estos de unos 150pb de media; la repetición del trinucleótido AAC en zonas codificantes da lugar a proteínas con largas regiones de asparaginas, glutaminas o treoninas; el espaciamiento intergénico posee un tamaño medio de 2,5kb, muy pequeño si lo comparamos con las 128kb presentes en humanos (Eichinger et al. 2005).

Biología celular y molecular de *Dictyostelium discoideum*

El funcionamiento y estructura celular de *Dictyostelium discoideum* es básicamente similar a la de cualquier célula eucariota de mamíferos.

La membrana plasmática de *Dictyostelium discoideum* debe tener una alta flexibilidad y fluidez para moverse por una amplia variedad de sustratos que componen el suelo de un bosque, además de poseer los mecanismos de reconocimiento necesarios para alimentarse de bacterias y levaduras. En contraposición a hongos como la levadura, *Dictyostelium* no posee una pared externa lo que posibilita el movimiento celular por pseudopodos y su forma de alimentación mediante fagocitosis.

El hábitat de *Dictyostelium discoideum* lo hace estar expuesto a rápidos cambios atmosféricos, los cuales pueden variar indirectamente la presión osmótica. El mecanismo de mantenimiento de unos valores óptimos de presión osmótica en mamíferos es totalmente diferente al de *Dictyostelium discoideum*. En mamíferos existe una regulación global a nivel corporal, de ello se ocupa el eje hipotálamo-hipófisis y el corazón que envían una serie de señales a los nefrocitos que son los encargados finales de mantener un balance adecuado en la excreción de líquidos y sales, por lo tanto no existe una especialización celular que permita una respuesta a variaciones en la presión osmótica. Sin embargo en *Dictyostelium discoideum* existe una red de cisternas y túbulos interconectados que se hinchan con agua en un ambiente hipoosmótico y se deshinchan en uno hiperosmótico (Du et al. 2008). La entrada de agua se produce por difusión mientras que la salida se da gracias a la conexión de la vacuola contráctil con la membrana plasmática, que de ese modo posibilita la salida por transporte activo.

El retículo endoplásmico en *Dictyostelium* es similar al de eucariotas superiores (Monnat et al. 1997), posee diferentes enzimas representativas de este orgánulo como PDI ("protein disulfide isomerase") o enzimas implicadas en la glicosilación proteica (West et al. 2004). El aparato de Golgi participa especialmente en la exocitosis masiva de proteínas durante la formación de la cubierta de las esporas (Takemoto, Yamamoto and Takeuchi 1985). Los núcleos son pequeños con pronunciados nucleolos e histonas muy similares a las de eucariotas superiores (Fisher 2001). Las

mitocondrias son muy parecidas en morfología y funcionamiento a las humanas (Barth, Le and Fisher 2007).

La captación de nutrientes en *Dictyostelium discoideum* se realiza por macropinocitosis en cultivo axénico mediante unas estructuras vesiculares de 0,6μm llamadas macropinosomas que internalizan el medio para su digestión intracelular (Maniak 2002). El crecimiento no axénico se da sobre un sustrato bacteriano de *Klebsiella aerogenes*, *Escherichia coli* u otras especies bacterianas, mediante fagocitosis. Ambos procesos son dependientes de una reorganización del citoesqueleto donde proteínas como actina, miosina o coronina son fundamentales (Maniak 1999), sin embargo la señalización celular que los inicia y regula es diferente. Por ejemplo, la cepa mutante en la subunidad β de proteínas G afecta a la fagocitosis mientras que no altera en absoluto la macropinocitosis, justamente al contrario de lo que ocurre con la cepa mutante de PI3K (Cardelli 2001). En ambos casos los endosomas pasan por varios procesos de fusión y fisión vesicular, acidificando su lumen y recibiendo diferentes enzimas lisosomales que degradarán su cargo. Posteriormente a este proceso el pH de los endosomas comienza a elevarse hasta neutralizarse. El proceso finaliza con la exocitosis del material no degradado y de algunos enzimas lisosomales al medio extracelular (Maniak 2003).

Los lisosomas son orgánulos derivados del sistema endomembranoso, en su formación intervienen el aparato de Golgi desde donde se produce la salida de estas vesículas con enzimas hidrolíticas procedentes del retículo endoplásmico rugoso.
Existen dos tipos de lisosomas en *Dictyostelium*, unos positivos a
N acetilglucosamina-1-fosfato que se fusionan con endosomas a los 3 minutos de la formación de éstos y otros positivos a manosa-6-fosfato que se fusionarán al cabo de 15 minutos. Al igual que en mamíferos constan de enzimas hidrolíticos y proteolíticos como lipasas, glucosidasas, proteasas y nucleasas (Fisher 2001). El pH lisosomal en *Dictyostelium* es de 3,5 (Marchetti, Lelong and Cosson 2009), ligeramente más ácido que en humanos donde alcanza 4,8 o levaduras con un valor de 6 (Ni et al. 2011).

Fase de desarrollo multicelular

Dictyostelium discoideum posee una etapa de vida unicelular en presencia de alimento y otra de desarrollo o pluricelular en ausencia de éste donde son fácilmente reconocibles varios estadios o etapas (Fig.3.). Son muchas las proteínas y

compuestos que regulan el paso de la etapa unicelular a la etapa pluricelular y es precisamente esta transición la que ha hecho que se conozca a estos organismos con el nombre de amebas sociales.

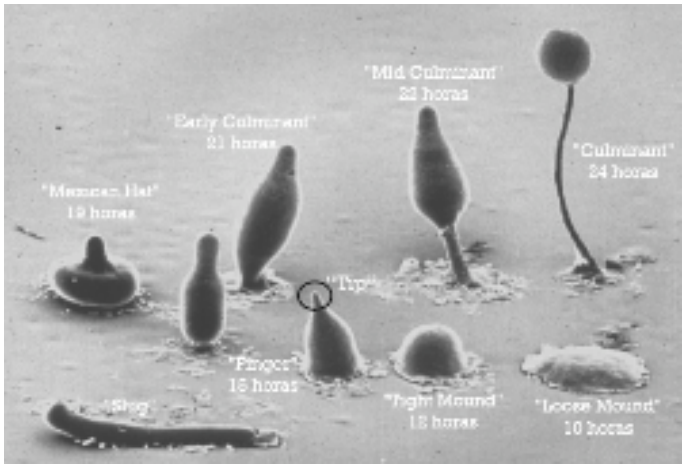


Fig.2. La ausencia de alimento activa en *Dictyostelium discoideum* un complejo programa de señalización y secreción proteica durante el c ual se suceden diferentes estadios morfológicos que finalizan en la formación de un cuerpo fructífero provisto de células tallo y esporas.

La disminución en la cantidad de nutrientes inicia el proceso a través de la respuesta al factor extracelular PSF ("pre-starvation factor") (Escalante and Vicente 2000). *Dictyostelium* requiere un número mínimo de 100000 células para iniciar la agregación celular y de ese modo síntetizar y secretar el factor CMF ("conditioned medium factor") (Gomer, Yuen and Firtel 1991). La presencia de estos 2 factores permite la síntesis y secreción autocrina de cAMP. Esta secreción se realiza de modo pulsatil generando un gradiente que permite a las amebas moverse quimiotácticamente hacia las zonas de mayores niveles de cAMP a través de una cascada de señalización que incluye receptores de 7 dominios transmembrana acoplados a proteínas G como los cARs ("cAMP receptors"). La cascada de señalización induce, vía PI3K, la síntesis PIP3 ("phosphatidylinositol-3,4,5-triphosphate") que de este modo quedará distribuído asimétricamente en la membrana plasmática (Comer and Parent 2002). Este claro gradiente de PIP3 da lugar a una reorganización del citoesquelto de actina y miosina y facilita el movimiento de las amebas unicelulares hacia el cAMP (Janetopoulos and Firtel 2008, Kolsch, Charest and Firtel 2008).

El movimiento coordinado de las amebas da lugar a la formación de un agregado celular o loose mound, el cual se recubre de una capa de polisacáridos y celulosa formando un agregado más compacto denominado “tight mound”. Formada esta estructura de células agregadas, debe iniciarse la diferenciación celular, esta etapa está íntimamente controlada por el factor de transcripción Gbf (“G-box binding factor”) que activa la expresión de los genes pre-tallo y pre-espora (Hjorth, Khanna and Firtel 1989).

En estadio de finger las células pre-tallo se dirigen a la zona anterior de la estructura y las pre-espora a la zona posterior. En este momento del desarrollo la estructura puede seguir dos caminos, la formación del cuerpo fructífero o por el contrario el paso por otra etapa intermedia llamada “slug”; la consecución de una u otra dependerá de factores como la luz o el pH del medio (Francis 1964). Este “slug” tiene capacidad migratoria ya que es fototáctico y termotáctico. Evolutiva y biológicamente la existencia de esta etapa “slug” es tremendamente útil para la supervivencia de *Dictyostelium*. El hábitat que rodea a *Dictyostelium discoideum*, tierra, hojas, piedras, puede dificultar la formación final de un cuerpo fructífero o puede disminuir ostensiblemente las probabilidades de que sus esporas alcancen otro hábitat más propicio para su futura supervivencia. Unos pocos centímetros pueden significar zonas de mayor cantidad de luz o temperatura y consecuentemente la formación del cuerpo fructífero en un lugar más adecuado para la dispersión de esporas.

En la etapa de culminación las células pre-tallo y pre-espora diferencian a célula tallo y espora respectivamente y migran hasta ocupar su localización final en el cuerpo fructífero. Las células tallo sufren un proceso de vacuolización y muerte celular que junto con la secreción de celulosa dan resistencia al tallo que debe sujetar las esporas (Fig.3.). Por su parte, las esporas secretan proteínas y celulosa que forman una compleja capa que las aisla del exterior y le permite sobrevivir largos periodos de tiempo en ausencia de nutrientes (Escalante and Vicente 2000, Early 1999).

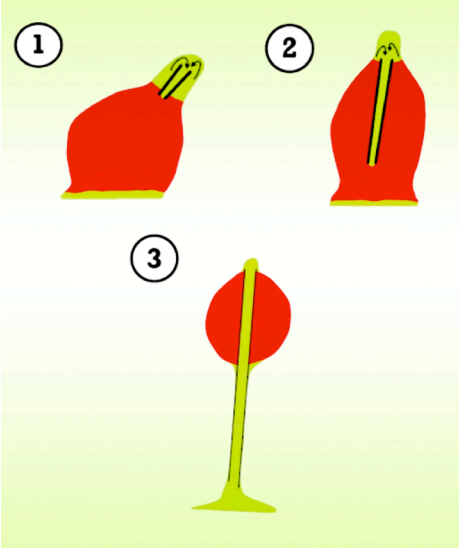


Fig.3. Migración de células pre-tallo (verde) y pre-esporas (rojo) previa a la formación del cuerpo fructífero. (1,2) Durante la culminación las células tallo comienzan a migrar hacia abajo para formar el tallo y el disco basal, sin embargo las pre-esporas son elevadas por el tallo en crecimiento para formar el esporocarpo, diferenciandose en esporas en la fase terminal del desarrollo. (3) Disco basal, tallo y esporocarpo totalmente formados en el estadio de cuerpo fructífero.

El correcto funcionamiento de la autofagia o de manera más concisa de la muerte celular por autofagia es fundamental para la diferenciación que da lugar a la formación de células tallo (Tekinay et al. 2006). Así por ejemplo cepas mutantes para el gen *atg1* ("autophagy-related gen 1"), una kinasa que activa autofagia, no son capaces de realizar un proceso autofágico correcto y no forman células tallo. Además es también necesaria la presencia en el medio extracelular de moléculas como DIF-1 ("differentiation inducing factor"), una hexafenona clorada (Kay and Jermyn 1983).
La diferenciación celular que da lugar a la formación de esporas está regulada por PKA ("cAMP-dependent protein kinase") y dos péptidos, SDF1 ("spore differentiation factor 1") y SDF2 ("spore differentiation factor 2") (Anjard et al. 1998a, Anjard et al. 1998b, Mann et al. 1994, Williams 2006).

Con la formación de un cuerpo fructífero se facilita la dispersión de las esporas, las cuales germinarán cuando las condiciones ambientales sean propicias dando lugar a nuevas amebas unicelulares.

Desde el punto de vista de la biología evolutiva la formación de este organismo pluricelular donde 20.000 amebas unicelulares mueren al diferenciarse a células tallo dando la posibilidad a otras 80.000 amebas que han diferenciado a esporas de colonizar otros hábitats que sean propicios para su germinación, representa un fascinante fenómeno de cooperación y altruísmo (Li and Purugganan 2011).

Dictyostelium discoideum como modelo experimental

La manipulación experimental de *Dictyostelium discoideum* es extremadamente sencilla. El crecimiento de *Dictyostelium*, es óptimo a 22ºC (muere a temperaturas superiores a 30ºC), pudiendo ser axénico o en presencia de bacterias (Fig.4.). La composición del medio axénico se caracteriza por ser rica en peptona, extracto de levadura, glucosa y sales minerales. El perfil de crecimiento en agitación es similar al de cualquier microorganismo, estableciéndose las fases de adaptación, de crecimiento exponencial, estacionaria y de muerte.

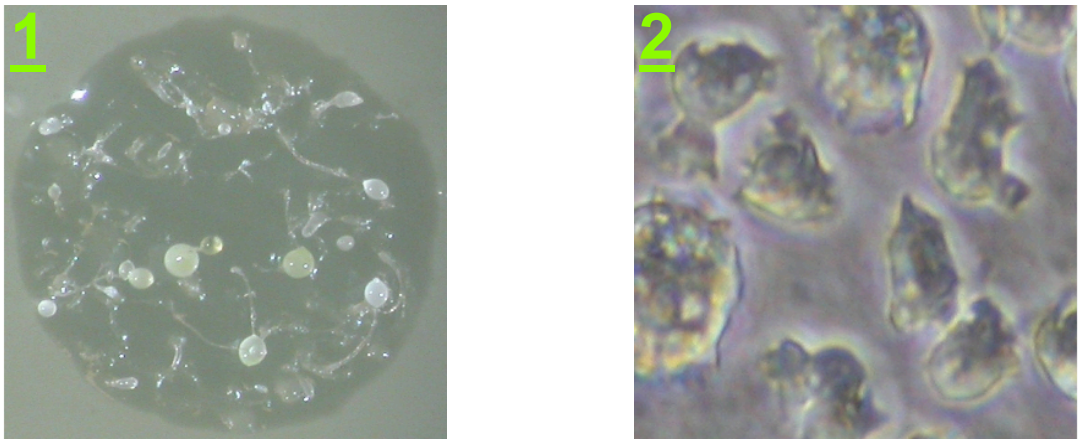


Fig.4. Células de *Dictyostelium* en crecimiento axénico y desarrollo multicelular en presencia de bacterias. (1) La zona más periférica es la de crecimiento, aquí no se observan cuerpos fructíferos ya que existen bacterias, sin embargo en la zona interior la ausencia de alimento activa el comienzo de la fase de desarrollo pluricelular de *Dictyostelium* con la consiguiente formación de cuerpos fructíferos. (2) Células de *Dictyostelium* en su etapa unicelular en presencia de alimento.

La haploidía y alta tasa de recombinación homóloga hacen de *Dictyostelium discoideum* un buen microorganismo para realizar interrupciones e inserciones génicas o sobreexpresion de genes mediate inserción de plasmidos (Torija, Robles and Escalante 2006a). Además también existe la posibilidad de realizar rastreos genéticos de supresión mediante los cuales se pueden identificar genes supresores de mutaciones preexistentes. Estos rastreos se pueden realizar mediante la transformación de la cepa mutante del gen en el que estemos interesados con genotecas de sobreexpresión de cDNA (Robinson and Spudich 2000) o mediante la técnica de REMI ("restriction enzyme mediated integration") (Loomis 1987), donde la interrupción de otro gen complementará el fenotipo de la cepa mutante. Por supuesto existe la posibilidad de usar fusiones con proteinas como GFP ("green fluorescent

protein") y también el uso de ARN de interferencia (Kuhlmann, Popova and Nellen 2006).

Dictyostelium discoideum como modelo en fisiopatología

La fácil manipulación y la similitud con humanos de muchos procesos presentes en *Dictyostelium discoideum* como diferenciación celular, autofagia, división celular, motilidad y quimiotaxis, fagocitosis y macropinocitosis, fototaxis y termotaxis, morfogénesis o interacción patógeno-huesped en estudios de infección, hacen de *Dictyostelium* un excelente modelo para el estudio molecular de diferentes patologías presentes en humanos. Por ello es usado en la actualidad por varios grupos de investigación como una herramienta más con la que ampliar el conocimiento sobre diferentes patologías (Escalante 2010).

Un ejemplo de esta aplicación es el uso del cisplatino, un agente quimioterapeútico que basa su mecanismo de acción en la unión de un átomo de platino al ADN de manera covalente, impidiendo su replicación y transcripción. La célula ante este daño celular pone en funcionamiento una serie de mecanismos de reparación de ADN, sin embargo ante la imposibilidad de reparar un daño tan drástico activa la ruta apoptótica. El uso de rastreos genéticos por REMI en *Dictyostelium* mostró la relevancia de los enzimas S-1-P-liasa ("Sphingosine-1-P-lyase"), esfingosina quinasa y ceramida sintasa en la resistencia a cisplatino. La modulación de los niveles relativos de los lípidos bioactivos S-1-P ("spingosine-1-phosphate") y ceramida regulan la respuesta a este droga en términos de supervivencia y muerte celular (Alexander and Alexander 2010).

La lisoencefalia es una enfermedad que afecta al desarrollo del cerebro en niños. Ésta es causada por mutaciones en los genes *lis1* y *dcx*. El único homólogo claro a *dcx* fuera del reino animal se encuentra en *Dictyostelium*. Estudios en *Dictyostelium* mostraron la importancia de Lis1 y Dcx en la unión del centrosoma al núcleo y en la integridad de la cromatina (Meyer, Kuhnert and Graf 2010).

Quizá la enfermedad donde mayor conocimiento se ha obtenido usando a *Dictyostelium* como modelo sea en el desorden bipolar. El grupo de Robin Williams ha realizado avances en los mecanismo de acción del ácido valproico y del litio, dos de las terapias clásicas utilizadas para paliar esta patología. Tanto el ácido valproico

(AVP) como litio disminuyen los niveles de InsP3 ("inositol 1,4,5-triphosphate"), los cuales están íntimamente ligados al desarrollo de esta enfermedad. Al igual que en el caso del cisplatino, el abordaje por REMI mostró un nuevo enzima con un importante papel en el efecto que el ácido valproico y el litio causaban en *Dictyostelium*. La disrupción del enzima PO ("prolil oligopeptidasa") disminuye la susceptibilidad celular al litio aumentando los niveles de InsP3. La importancia de este hallazgo se apoya en el aumento de la actividad de este enzima en pacientes que sufren desorden bipolar (Ludtmann, Boeckeler and Williams 2010).

Dictyostelium también es usado en el estudio de otras patologías como enfermedades mitocondriales (Francione et al. 2010), síndrome de Strumpel (Carnell and Insall 2010), síndrome de Diamond-Bodian-Shwachman, estudios de interacción patógeno huésped en infección con microorganismos (Lima, Lelong and Cosson 2010, Steinert 2010) o en la relación de los cuerpos de Hirano con enfermedades neurodegenerativas (Kim et al. 2009).

Autofagia

El equilibrio entre la síntesis y degradación proteica, la eliminación de proteínas con un plegamiento incorrecto para impedir la formación agregados proteicos, la necesidad de una fuente de nitrógeno para la síntesis de nuevos aminoácidos en periodos de ayuno, el recambio de orgánulos citoplásmicos, la respuesta a procesos de stress celular, la lucha contra patógenos intracelulares, todos estos procesos son esenciales para la supervivencia celular y dependientes de un correcto funcionamiento de la autofagia (Calvo-Garrido and Escalante 2010).

De modo coloquial la autofagia ha sido comparada a una escoba que transporta hasta los lisosomas diferentes cargos citoplásmicos para ser degradados (Cuervo 2008). Además de tener como sustrato mayoritario proteínas, la autofagia también regula los niveles intracelulares de "lipid droplets" (almacenes lipídicos de triglicéridos y colesterol) (Singh et al. 2009) y por ello ha sido involucrada también en obesidad.

Tipos de autofagia

Existen 3 tipos diferentes de autofagia en mamíferos (Fig.5.), microautofagia, macroautofagia y autofagia mediada por chaperona (AMC). La más conocida y estudiada de las 3 es la macroautofagia.

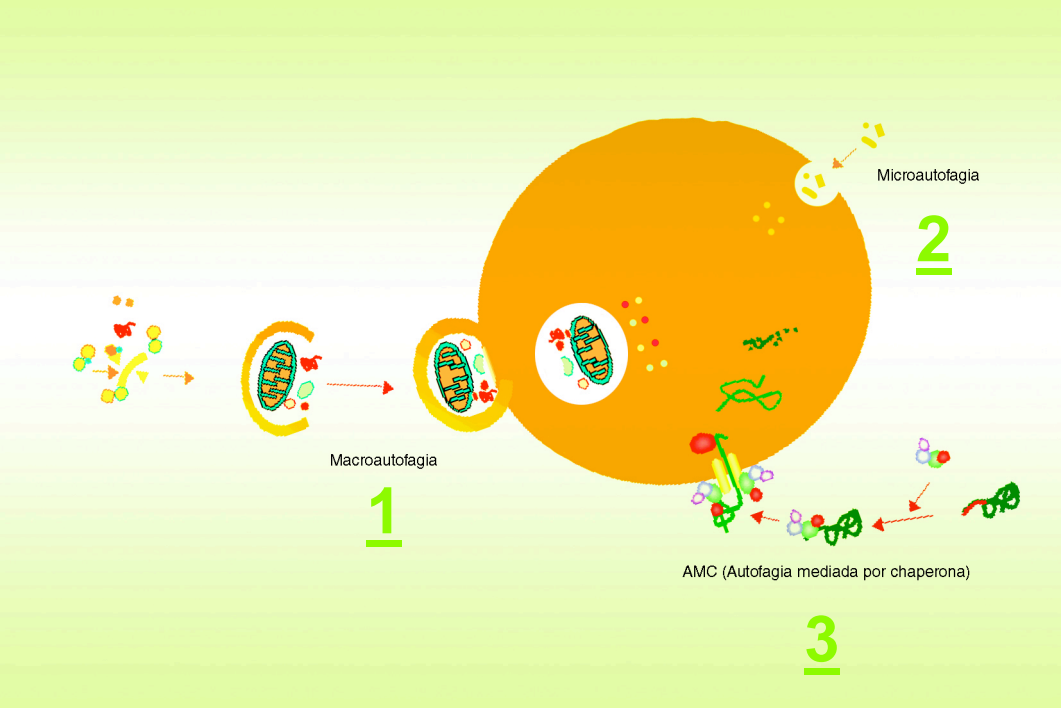


Fig.5. Representación esquemática de los 3 tipos diferentes de autofagia. (1) En la **macroautofagia** una vesícula de doble membrana engloba un cargo citoplásmico. Esta vesícula llamada autofagosoma se fusionará en última estancia con la membrana lisosomal lo que facilita la llegada del cargo citoplásmico al interior lisosomal para su degradación. (2) En la **microautofagia** la entrada del cargo citoplásmico al interior lisosomal se produce mediante una invaginación de la membranadel lisosoma. (3) Por último en la **AMC**, son un g rupo de chaperonas las encargadas de reconocer y transportar hasta la membrana lisosomal a prot eínas que poseen en su secuencia el mot ivo KFERQ, a llí un receptor de membrana reconocerá estas proteínas y facilitará su entrada al interior del lisosoma.

Macroautofagia

La macroautofagia se caracteriza por la formación, maduración y fusión con lisosomas de una vesícula de doble membrana que engloba diferentes cargos citoplásmicos (Klionsky 2005).

Esta doble membrana consta de una membrana interior, que será degradada al igual que el cargo autofágico en el lisosoma, y una externa que se fusionará con la membrana del lisosoma (Deretic 2008).

El origen de la doble membrana macroautofágica permanece aún sin dilucidar (Tooze and Yoshimori 2010), siendo varias las hipótesis acerca de su origen. El origen a partir de orgánulos específicos como el retículo endoplásmico (Axe et al. 2008) o mitocondrias (Hailey et al. 2010) es el que cuenta con un mayor respaldo, sin embargo también se hipotetiza con la posibilidad de un origen *de novo* o con un origen a partir de otros orgánulos celulares como el peroxisoma (Farre et al. 2010) o el aparato de Golgi (Ohashi and Munro 2010), incluso a partir de la membrana plasmática (Ravikumar et al. 2010).

Al igual que el origen de la membrana autofágica, la posible selectividad en la elección del cargo autofágico es un amplio tema de debate. Se han descrito casos de mitofagia (degradación selectiva de mitocondrias) (Bhatia-Kissova and Camougrand 2010), retículofagia (degradación selectiva de retículo endoplásmico) (Bernales, Schuck and Walter 2007), ribofagia (degradación selectiva de ribosomas) (Kraft and Peter 2008), pexofagia (degradación selectiva de peroxisomas) (Dunn et al. 2005) donde la macroautofagia estaría perfectamente orientada y regulada hacia la degradación parcial o total de parte de estos orgánulos, sin embargo también está muy documentado el carácter no selectivo de degradación de la autofagia en periodos de ayuno (Mizushima 2005).

Se conoce parcialmente la señalización y el modo en que estas membranas se estructuran, maduran y fusionan con el lisosoma durante la macroautofagia, pudiendo dividirse el proceso en 4 etapas diferentes (Calvo-Garrido et al. 2010):

Inducción: señales tan variadas como el ayuno, el stress celular (Kaushik, Singh and Cuervo 2010) o la presencia de patógenos intracelulares inician una cascada de señalización regulada por el complejo Atg1 (“autophagy related gen 1”). Este complejo está formado entre otras proteínas por Atg101, Atg13, FIP200 y la propia Atg1 y es regulado por el complejo TORC1 (“Target of rapamycin complex I”).

Nucleación: la señalización inicial da paso al ensamblaje de una membrana *de novo* o a la especialización de una región de membrana específica de algún orgánulo celular (retículo endoplásmico, mitocondria, peroxisoma entre otros) para la formación del fagóforo (estructura primaria de la formación del autofagosoma). El complejo PI3K es el encargado de conducir este proceso, proteínas como Bif1, UVRAG (“UV resistance-associated gen”), Vps34, Vps15 forman parte de él (Backer 2008).

Expansión vesícular: el fagóforo comienza a madurar, recoge su cargo citoplásmico y forma la vesícula completa o autofagosoma. Este proceso es dependiente de la señalización llevada a cabo por los complejos de conjugación similares a ubiquitina (Geng and Klionsky 2008) Atg5-Atg12 y Atg8, donde Atg5 se conjuga con Atg12 y Atg8 se une a la membrana del autofagosoma en formación mediante su conjugación a fosfatidiletanolamina. Otras proteínas que forman parte de estos dos complejos son Atg7, Atg10, Atg16, Atg3 y Atg4. Es importante destacar, debido a su importancia, que LC3 es la proteína humana homóloga a la proteína de levaduras Atg8, a su forma no lipidada se le conoce como LC3I y a la lipidada con fosfatidiletanolamina como LC3II.

Fusión del autofagosoma con el lisosoma: la fusión del autofagosoma con lisosomas maduros forma el autofagolisosoma, de este modo el cargo puede ser ya degradado.
La degradación del cargo autofágico y de la membrana interna del autofagosoma es seguida de la salida de los productos resultantes mediante permeasas lisosomales al citoplasma.

La amplia variedad de funciones celulares de la macroautofagia hace que su disfunción provoque, sea causa o la relacione con múltiples patologías como Parkinson, Alzheimer, Huntington (Banerjee, Beal and Thomas 2010), enfermedades lisosomales, cáncer (Abedin et al. 2007) o enfermedad de Crohn (Brest et al. 2010) y por ello es una diana terapeútica potencialmente excelente.

Autofagia mediada por chaperona

En la AMC un grupo de chaperonas en la que predomina la chaperona Hsc70 (“heat shock cognate 70 protein”) actúan como transportadores de proteínas hacia los lisosomas. Las proteínas transportadas deben tener un motivo KFERQ en su secuencia aminoacídica para ser degradadas o al menos este motivo debe estar formado por un aminoácido básico, otro ácido, un tercero hidrofóbico, el cuarto puede ser tanto básico como hidrofóbico pero nunca ácido y siempre uno de los extremos debe tener una glutamina o asparagina. En la membrana lisosomal se encuentran anclados monómeros del receptor Lamp2a (“Lysosomal-associated membrane protein 2a”) y la llegada e interacción de la proteína cargo con Lamp-2a activa su dimerización. La proteína cargo ayudada de Hsc70 despliega su estructura permitiendo su entrada al interior lisosomal a través de Lamp2a (Bejarano and Cuervo 2010).

Poco o nada es conocido acerca de las rutas de señalización que controlan la AMC, sin embargo si que parece existir una interelación entre la macroautofagia y la AMC.
La macroautofagia es el mecanismo que mayoritariamente se encuentra degradando y suministrando a las células los aminoácidos necesarios para la síntesis de nuevas proteínas durante el ayuno, sin embargo a partir de las 6 horas su funcionamiento decae ostensiblemente. En su lugar es la AMC el mecanismo fundamental encargado de los procesos degradatorios asociados al ayuno, alcanzando su mayor actividad 20 horas después del inicio de éste (Kaushik et al. 2008).

No se ha descrito la existencia de AMC en *Dictyostelium discoideum* o levaduras ya que no se han encontrado homólogos del receptor lisosomal Lamp2a. En el caso de existir debe implicar la existencia de otros receptores no identificados.

Microautofagia

La microautofagia es la menos conocida de los 3 tipos de autofagia y prácticamente toda su información proviene de los estudios en la levadura *Saccharomyces cerevisiae*. La microautofagia se caracteriza por la formación de una invaginación de la membrana lisosomal en mamíferos y vacuolar en levaduras que engloba diferentes cargos autofágicos para su degradación (Deretic 2008).

Macroautofagia en mamíferos, *Dictyostelium discoideum* y *Saccharomyces cerevisiae*: similitudes y diferencias

El conocimiento de la autofagia en levaduras como *Saccharomyces cerevisiae* es muy amplio, no así en *Dictyostelium discoideum* donde en estos momentos sólo podemos hablar de algunos estudios fenotípicos de cepas deficientes en los genes homólogos de *atg1*, *atg5*, *atg6*, *atg7* y *atg8* y cierta similitud en algunos procesos como más tarde veremos (Otto et al. 2004). Sin embargo la mayor similitud a nivel molecular y celular de la autofagia entre *Dictyostelium* y mamíferos hacen que sea un modelo muy relevante para su estudio.

En la levadura *Saccharomyces cerevisiae* la macroautofagia puede tener un carácter biosintético en vez de degradativo, se trata de la denominada ruta Cvt (“cytoplasm to

vacuole targeting"). En esta ruta el autofagosoma transporta a la vacuola la enzima hidrolasa aminopeptidasa I, un enzima residente en la vacuola. La macroautofagia comparte para su funcionamiento muchas proteínas implicadas en esta ruta Cvt, sin embargo su regulación y funcionalidad son diferentes (Huang and Klionsky 2002).
Fue el estudio de esta ruta lo que reportó muchos de los genes relacionados con macroautofagia, denominados genes atg por "autophay-related genes". Gran parte de ellos encontraron posteriormente un claro homólogo en mamíferos. Un estudio detallado de los homólogos en *Dictyostelium*, humanos y levadura se muestra en uno de los artículos que se publicaron durante esta tesis (Calvo-Garrido et al. 2010).

Sin embargo existen ciertas salvedades que diferencian la ruta macroautofágica de levaduras, mamíferos y *Dictyostelium*. En levaduras existe una gran vacuola citoplásmica, en vez de múltiples lisosomas como en mamíferos y *Dictyostelium*. Solo existe una zona de nucleación de autofagosomas en levaduras llamada sitio preautofagosómico, sin embargo en mamíferos y *Dictyostelium* existen multiples focos de formación de autofagosomas (Calvo-Garrido and Escalante 2010). Es de esperar que esta similitud estructural se traduzca a nivel molecular en la existencia de proteínas homólogas presente en *Dictyostelium* y mamíferos y ausentes en levadura. Dos de estas proteínas son Atg101 (Hosokawa et al. 2009) y Vmp1 ("Vacuole membrane protein 1"). Gran parte de esta tesis está basada en el estudio de esta proteína Vmp1 y su relación con autofagia.

Vmp1

La relación de Vmp1 con macroautofagia es un resultado posterior al inicio de esta investigación, por ello la primera pregunta que debemos responder es por qué se decidió investigar la función molecular de Vmp1 y las razones para utilizar *Dictyostelium* en esta investigación.

Vmp1, una proteína de función desconocida presente en *Dictyostelium discoideum*

La respuesta la encontramos en el desconocimiento de la función de muchos de los genes que los estudios de secuenciación del genoma humano hallaron en 2003 (Venter et al. 2001). Esta formidable investigación supuso la secuenciación de todo el genoma humano con una estimación cercana a los 25000 genes, abriendo la posibilidad de aplicar estudios comparativos con otros genomas que pudieran ser secuenciados. Uno de ellos fue el de *Dictyostelium discoideum* (Eichinger et al. 2005), por ello se abordó el estudio de genes conservados de función desconocida en en este sistema modelo (Torija et al. 2006a, Torija et al. 2006b).

Estos estudios previos de genómica comparativa en nuestro laboratorio revelaron la existencia de 41 proteínas homólogas entre *Dictyostelium discoideum* y humanos que no estaban presentes en las levaduras *Saccharomyces cerevisiae* y *Schizosaccharomyces pombe* y que además no poseían ningún dominio funcional conocido (Torija et al. 2006a). Se obtuvieron cepas mutantes por disrupción génica de 28 de estos genes, uno de estos 28 genes interrumpidos era DDB0234044, el cual mostraba una alta homología con la proteína humana Vmp1 (Torija et al. 2006a). Estudios morfológicos preliminares de la cepa K.O. ("knock out") de *vmp1* arrojaron una serie de resultados muy interesantes y por ello se entendió que podía ser objeto de un estudio mucho más pormenorizado y extenso.

Vmp1 en otros sistemas e implicaciones patológicas

Vmp1 es una proteína de 406 aminoácidos con 6 u 8 posibles regiones transmembrana que varían dependiendo de la predicción bioinformática utilizada. Los datos existentes en el inicio de la investigación sobre Vmp1 son esencialmente descriptivos y están relacionados con sus niveles de expresión en diferentes tejidos, especialmente en páncreas (Dusetti et al. 2002).

Vmp1 fue hallado como un gen sobreexpresado dentro de un estudio global de expresión génica en ratas a las que se les provocaba pancreatitis aguda mediante la inyección de ceruleína (Dusetti et al. 2002). Resultados similares fueron obtenidos posteriormente en pancreatitis crónica e inducciones de pancreatitis aguda con L-

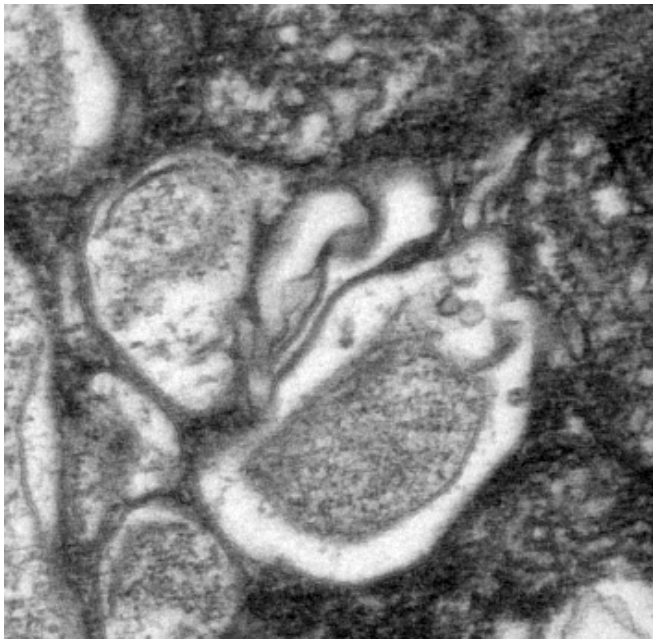
arginina (Vaccaro et al. 2003, Jiang et al. 2004). *Vmp1* se expresa en páncreas exclusivamente en células acinares, no haciéndolo en otros tipos celulares pancreáticos. En estado embrionario a 17 días pre-parto muestra su máxima expresión, la cual comienza paulatinamente a decaer hasta que a día 25 post-parto no se observa.

También se expresa en otros tejidos como placenta, cerebro, timo, hígado o retina, no estando necesariamente asociada su expresión con ninguna patología, sin embargo en riñón bajo un daño isquémico transitorio se observa un sobreexpresión similar a la ocurrida en pancreatitis aguda (Dusetti et al. 2002).

La sobreexpresión de *vmp1* en células Cos7 induce la formación de vacuolas citoplásmicas y la muerte celular 48 horas más tarde. En éstas células la proteína de fusión Vmp1-GFP localiza en regiones cercanas al aparato de Golgi, al retículo endoplásmico y en las membranas de las vacuolas que se forman como consecuencia de su sobreexpresión (Vaccaro et al. 2003) .

Es importante destacar además que Vmp1, como muchos otros genes, ha sido encontrado desregulado en diferentes procesos cancerígenos (X. et al. 2002).

Los estudios realizados durante esta tesis doctoral han permitido generar una cepa de falta de función de Vmp1 en *Dictyostelium*, una amplia caracterización de su fenotipo asi como su relación con macroautofagia y la caracterización preliminar de este proceso en *Dictyostelium discoideum*.



OBJETIVOS

OBJETIVOS

Estudio de la función de la proteína Vmp1 en *Dictyostelium discoideum*

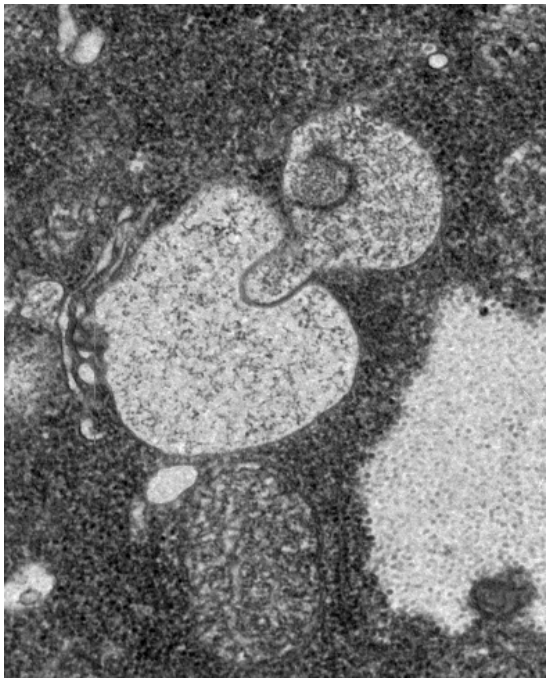
1. Localización subcelular de la proteína Vmp1.

2. Generación y análisis fenotípico de una cepa deficiente en *vmp1*.

3. Estudio de la relación de la proteína Vmp1 con autofagia.

Caracterización de la autofagia en el microorganismo *Dictyostelium discoideum*

1. Análisis de genes conservados y puesta a punto en *Dictyostelium discoideum* de técnicas y marcadores de autofagia.



ARTÍCULOS PUBLICADOS

CAPÍTULO 1: VACUOLE MEMBRANE PROTEIN 1 IS AN ENDOPLASMIC RETICULUM PROTEIN REQUIRED FOR ORGANELLE BIOGENESIS, PROTEIN SECRETION, AND DEVELOPMENT

Vmp1 (Vacuole membrane protein 1) es una proteína de función desconocida sin motivos funcionales reconocibles presente en *Dictyostelium discoideum*, ausente en levaduras como *Saccharomyces cerevisiae* y *Schizosaccharomyces pombe* y con un claro homólogo en humanos. Vmp1 ha sido relacionado con diferentes procesos fisiopatológicos como pancreatitis aguda y cáncer. En el primero de éstos, *vmp1* se encuentra sobreexpresado, mientras que en el segundo, en cáncer, y de un modo más específico, cáncer de riñón, se expresa en menor cantidad en tumores metastáticos respecto a tumores primarios, lo cual parecía indicar algún tipo de papel de Vmp1 en la capacidad invasiva o metastática del tumor.

Generamos una disrupción génica de *vmp1* en *Dictyostelium* para analizar su función. La expresión de la proteína de fusión Vmp1-GFP complementaba el fenotipo mutante y nos permitió determinar su localización subcelular en retículo endoplásmico mostrando una clara colocalización con el marcador de retículo PDI.

La cepa deficiente en *vmp1* en *Dictyostelium discoideum* muestra un fenotipo pleitrópico con un crecimiento prácticamente nulo en cultivo axénico y reducido en bacterias. También la biogénesis de la vacuola contráctil se encuentra impedida como muestran el patrón de dos marcadores de vacuola contráctil como rh50 y dajumin-GFP. Como consecuencia de esto, células expuestas a soluciones de baja osmolaridad explotan ante la incapacidad de revertir esta situación mediante el bombeo de agua al exterior celular. Otros orgánulos como el retículo endoplásmico o el aparato de Golgi presentan un morfología aberrante

La macropinocitosis, la fagocitosis y la secreción proteica convencional muestran también un funcionamiento deficiente.
También la entrada en la etapa de desarrollo de la cepa mutante de *vmp1* se encontraba impedida y muchos de los genes que se activan para ello no lo hacían.

Por primera vez observamos la presencia de estructuras autofágicas aberrantes en la cepa mutante de *vmp1* abriéndose de ese modo el camino hacia futuras investigaciones que relacionasen *vmp1* y autofagia.

Se estudió la posible conservación funcional de la proteína Vmp1 durante la evolución mediante un ensayo de complementación fenotípica. El fenotipo de células mutantes de *vmp1* en *Dictyostelium discoideum* fue complementado por la proteína Vmp1 de rata fusionada a GFP. Esto implica una posible conservación funcional a lo largo de la evolución de Vmp1.

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Vacuole Membrane Protein 1 Is an Endoplasmic Reticulum Protein Required for Organelle Biogenesis, Protein Secretion, and Development

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Vacuole membrane protein 1 (Vmp1) is membrane protein of unknown molecular function that has been associated with pancreatitis and cancer. The social amoeba *Dictyostelium discoideum* has a *vmp1*-related gene that we identified previously in a functional genomic study. Loss-of-function of this gene leads to a severe phenotype that compromises *Dictyostelium* growth and development. The expression of mammalian Vmp1 in a *vmp1*$^-$ *Dictyostelium* mutant complemented the phenotype, suggesting a functional conservation of the protein among evolutionarily distant species and highlights *Dictyostelium* as a valid experimental system to address the function of this gene. *Dictyostelium* Vmp1 is an endoplasmic reticulum protein necessary for the integrity of this organelle. Cells deficient in Vmp1 display pleiotropic defects in the secretory pathway and organelle biogenesis. The contractile vacuole, which is necessary to survive under hypoosmotic conditions, is not functional in the mutant. The structure of the Golgi apparatus, the function of the endocytic pathway and conventional protein secretion are also affected in these cells. Transmission electron microscopy of *vmp1*$^-$ cells showed the accumulation of autophagic features that suggests a role of Vmp1 in macroautophagy. In addition to these defects observed at the vegetative stage, the onset of multicellular development and early developmental gene expression are also compromised.

INTRODUCTION

One of the hallmarks of eukaryotic cells is the presence of complex intracellular membrane-bound organelles dedicated to specific functions. Part of the structural and functional specificity of these organelles is based on their distinct complement of proteins and membrane lipids. The link between membrane traffic, protein traffic, and organelle biogenesis is now becoming evident in the context of the secretory pathway (Derby and Gleeson, 2007). Failure of any component within the pathway can lead to abnormal targeting of proteins and membrane components that are necessary for organelle function or biosynthesis (Howell *et al*., 2006). Furthermore, some of these defects in organelle biogenesis have been found to be associated with human diseases (Dhaunsi, 2005).

Vacuole membrane protein 1 (Vmp1) is a conserved putative membrane protein with no recognizable functional motifs. The function of Vmp1 is now beginning to be elucidated. Several lines of evidence suggest a possible role of this protein in membrane traffic and organelle organization. It has been described as a stress-induced endoplasmic reticulum (ER) protein in the rat exocrine pancreas that is highly expressed during acute pancreatitis (Dusetti *et al*., 2002; Vaccaro *et al*., 2003). Overexpression of this protein in cell culture leads to vacuole formation and cell death, a process that is observed in pancreatitis (Dusetti *et al*., 2002). A recent report also identified Vmp1 as a novel autophagy-related membrane protein involved in mammalian pancreatitis-induced autophagy (Ropolo *et al*., 2007). In *Drosophila*, Vmp1 (known as TANGO-5) was also identified in a functional genomic screen by using RNA interference. TANGO-5 was found to be required for protein secretion and Golgi organization (Bard *et al*., 2006). In another study, Vmp-1 was localized in the plasma membrane in the kidney cancer cell line Caki-2, and it was found to be essential for cell–cell contact (Sauermann *et al*., 2008). These results suggested a totally different function of Vmp1 in tumor cells. Therefore, the function of this protein remains controversial and seems to depend on the specific cell type studied.

Vmp1 is a conserved protein and the study of its complex function might benefit from the use of simple experimental systems such as *Dictyostelium discoideum*. Using the completed *Dictyostelium* genome sequence, we generated a collection of mutants by targeted disruption of genes with unknown function that are highly conserved between *Dictyostelium* and humans, but also absent from the genomes

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Abbreviations used: CV, contractile vacuole; ER, endoplasmic reticulum; GFP, green fluorescence protein; PDI, protein disulfide isomerase; VMP1, vacuole membrane protein 1; TEM, transmission electron microscopy.

of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Torija *et al*., 2006a,b). Among those genes, we identified Vmp1 (named DupF in that analysis). Its absence in any fungi makes *Dictyostelium* the simplest genetically tractable model system to address its function.

Dictyostelium is a eukaryotic microorganism used as a model to study basic cellular processes, including membrane traffic and the endocytic pathway (Maniak, 2003). These social amoebae live as solitary cells feeding on other microorganisms by phagocytosis. Laboratory strains are also capable of growth in axenic media that is taken up by macropinocitosis. The vacuoles of ingested material fuse with lysosomes and undigested residues are secreted by exocytosis. As in many soil microorganisms, water regulation is essential for survival. A specialized organelle, the contractile vacuole (CV) system, is composed of an independent network of membrane tubules and cisternae that fill up and expel water by transient fusion with the plasma membrane (Gabriel *et al*., 1999). Contractile vacuole biogenesis is dependent on clathrin-coated vesicles and the adaptor-protein complex 1 (AP-1) for transporting protein and membranes required for the CV formation (O'Halloran and Anderson, 1992; Lefkir *et al*., 2003). As a result, defects in AP-1 function lead to impaired osmoregulation.

Besides its interest as a cellular model, *Dictyostelium* has the exceptional ability to form a multicellular organism by aggregation of solitary cells. The differentiation program is triggered by starvation and leads to the formation of a fruiting body composed of spores supported by a stalk (Escalante and Vicente, 2000).

In this report, we describe the first loss-of-function mutation for a Vmp1 homologue in a model system. We have found that Vmp1 is an endoplasmic reticulum protein in *Dictyostelium* necessary for the integrity of this organelle. The lack of this ER protein has pleiotropic defects in several membrane traffic-dependent processes such as organelle biogenesis and structure, endocytosis, and protein trafficking. Our results also suggest that an aberrant pattern of protein secretion during starvation might in part account for the impairment in the transition from growth to development in *Dictyostelium*.

MATERIALS AND METHODS

Dictyostelium *Cell Culture, Transformation, and Development*

Cells were grown axenically in HL5 medium or in association with *Klebsiella aerogenes* in SM plates (Sussman, 1987). Transformations were carried out by electroporation as described previously (Pang *et al*., 1999). For synchronous development, axenically growing cells were washed from culture media by centrifugation, resuspended in PDF buffer (20 mM KCl, 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$, pH 6.4) and deposited on nitrocellulose filters (Shaulsky and Loomis, 1993). Because Vmp1 mutant cells do not grow well in axenic HL5 media the strain was initially grown in association with bacteria. For most of the experiments (unless otherwise indicated), ~10,000 cells were mixed with *Klebsiella* (300 μl of an overnight culture) and plated in SM plates. After 3 d, the clear lawn of cells were taken from the plate and used directly or resuspended in HL5. The remaining bacteria were then washed out by centrifugation, and the cells were deposited again in an appropriate volume of HL5 during an overnight, unless otherwise indicated. For filter development mutant cells were taken from SM plates as indicated above, washed, and deposited on the filters. Mutant cells in HL5 or PDF remain viable, and no cell lysis occurs in these conditions.

Generation of Mutant Strains

Disruption of Vmp1 gene in *Dictyostelium* was performed as described previously (Torija *et al*., 2006a). Briefly, DNA fragments ranging from 2 to 2.5 kb containing the genes to be disrupted were cloned by polymerase chain reaction (PCR) from genomic DNA. Insertion of the blasticidin resistance cassette by in vitro transposition by using the vector was performed as described previously (Abe *et al*., 2003). The constructs containing the flanking regions and the transposon were amplified by PCR, and the products were transformed in *Dictyostelium* cells by electroporation. Transformants were plated in association with bacteria for clonal isolation and screened for homologous recombination by PCR by using oligonucleotides surrounding the site of insertion. The DNA of the transformant clones used for PCR was isolated from cells of the growing zone by using the Master amp DNA extraction solution from EPICENTRE.

Gene Expression Analysis, Northern Blots, and Western Blots

RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. The different RNAs were separated by electrophoresis, transferred to nylon membranes, and hybridized to the indicated radioactive-labeled PCR probes. All DNA/RNA manipulation and Western blot analysis were performed according to methods described previously (Ausubel *et al*., 1992).

Transmission Electron Microscopy

Wild-type (WT) and mutant cells were incubated in Petri dishes with HL5 overnight to allow the attachment of the cells. The media were discarded, and cells were rapidly fixed with 1.25% glutaraldehyde in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 6.8, containing 1% sucrose and 2 mM Mg_2SO_4 for 60 min at 37°C. Cells were then gently scraped, pelleted at 200 × *g*, rinsed in PIPES buffer (3 times), postfixed with 1% (wt/vol) OsO_4, 1% (wt/vol) $K_3Fe(CN)_6$ in PIPES buffer for 1 h at room temperature in the dark, and rinsed in PIPES buffer. Cells were treated for 5 min with 0.1% tannic acid in PIPES buffer, dehydrated with graded ethanol solutions, and finally embedded in Epon plastic resin. Ultrathin sections were stained with 2% uranyl acetate for 30 min, and then with lead citrate for 10 min and observed with a JEOL 1010 transmission electron microscope operating at 80 kV with a Gatan BioScan model 792 module for acquisition of digital images with Digital. Micrograph 3.4.3 acquisition software (Gatan, Pleasanton., CA). ImageJ 1.37 software (National Institutes of Health, Bethesda, MD) was used for the morphometric analysis. Data are mean values with SD.

Endocytosis, Exocytosis, and Phagocytosis

Wild-type cells were grown axenically in HL5 and mutant cells were initially grown in SM-plates and then incubated overnight in HL5 before being used for the experiments. Endocytosis, exocytosis, and phagocytosis of fluorescent markers were performed according to Rivero and Maniak, 2006). Results are shown as mean values with SD from duplicates or triplicates of at least three independent experiments. Significance of differences between groups was determined by Student's *t* test.

Conditioned Media, In-Gel Digestion of Proteins, Matrix-Assisted Laser Desorption Ionization-Tandem Mass Spectrometry (MALDI-MS/MS), and Database Searching

Conditioned media were obtained by the incubation of cells in PDF at a concentration of 1 × 10E7 cells/ml during 7 h in shaking culture. The media were washed free of cells by centrifugation at 1000 rpm for 5 min. Conditioned media were subsequently used for the biological experiments and for analysis by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis the gel was silver stained with the silver staining kit from GE Healthcare (Chalfont St. Giles, United Kingdom), according to the instructions that allow the subsequent identification by MALDI-MS/MS. Differential bands between wild type and mutant were excised manually from the gel and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol used was that described previously (Shevchenko *et al*., 2006), with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (GE Healthcare) in 50 mM ammonium bicarbonate (99.5% purity; Sigma-Aldrich, St. Louis, MO) and alkylation with 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI) at a final concentration of 8 ng/μl in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma-Aldrich) was added for peptide extraction.

An aliquot of the above-mentioned digestion solution was mixed with an aliquot of cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChip prestructured MALDI probe (Bruker Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained in an automated analysis loop using an Ultraflex time-of-flight (TOF) mass spectrometer (Bruker Daltonics) equipped with a LIFT MS/MS device (Suckau *et al*., 2003). Spectra were acquired in the positive-ion mode at 50-Hz laser frequency, and 100-1000 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precur-

sors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Automated analysis of mass data was performed using the flexAnalysis software (Bruker Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z 842.510 and m/z 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. MALDI-MS and MS/MS data were combined through the BioTools program (Bruker Daltonics) to search nonredundant protein databases (National Center for Biotechnology Information, Bethesda, MD; and SwissProt, Swiss Institute for Bioinformatics, Switzerland) by using the Mascot software (Matrix Science, London, United Kingdom) (Perkins *et al*., 1999).

Green Fluorescent Protein (GFP) Expression Constructs, Immunocytochemistry, and Microscopy

The *Dictyostelium vmp1* gene was amplified from genomic DNA by using oligonucleotides containing targets for the restriction enzyme BamH1 and XbaI. The fragment was cloned in pGEMt-easy vector and sequenced to check for possible polymerase errors. The fragment was subsequently cloned into the BamH1 and XbaI sites of the GFP vector pDV-CGFP-CTAP, kindly provided by Pauline Shaap (University of Dundee, Dundee, United Kingdom). The construct, driven by actin15 promoter, contained the complete Vmp1 coding region fused to GFP-TAP. A similar approach was used for cloning the rat Vmp1 vector. In this case, the complete Vmp1 coding region was obtained by reverse transcription (RT)-PCR from RNA isolated from rat tissue.

For immunocytochemistry, WT and mutant cells (incubated previously in HL5 overnight), were allowed to adhere to coverslips and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After two washes with PBS cells were permeabilized with chilled methanol during 2 min and incubated during 20 min in blocking buffer (0.2% bovine serum albumin in PBS). The samples were then incubated with the first antibody in blocking buffer for 1 h. After six washes with blocking buffer the appropriate secondary antibody (labeled with red Alexa 546) was added at a dilution of 1/1000 in blocking buffer for 30 min. After two washes with blocking buffer cells were mounted for microscopic observation. Confocal analysis was performed on a Leica TCS SP5 by using a PL APO 63×/1.4-0.6 objective and a LAS-AF (Leica Application Suite; Lecia, Wetzlar, Germany) software. For excitation of GFP a 488-nm argon laser was used. For fluorescence microscopy, an Olympus DP70 microscope with a Plan Neofluar 100×, 1.30 oil objective was used. The acquisition software was DP controller 2002, Olympus Optical CO.LTD. The antibodies and the dilution used for each were as follows: PDI (221-64-1 ascitis, mouse monoclonal) used at 1:1000; p80 (H161, mouse monoclonal) used at 1:10; Rh50 (rabbit polyclonal), used at 1:500. These antibodies were kindly provided by Pierre Cosson from the University of Geneva (Geneva, Switzerland). VatA (221-35-2 ascitis, mouse monoclonal) used a dilution of 1:3. Kindly provided by Marcus Maniak (Kassel University, Kassel, Germany). AprA (rabbit polyclonal) used at 1:1000. Kindly provided by Richard Gomer (Rice University, Houston, TX).

RESULTS

Disruption of Vmp1 in Dictyostelium Leads to a Severe Defect in Osmoregulation

Dictyostelium vmp1 (DDB0234044) codes for a putative transmembrane protein of 403 amino acids. The level of identity between the human and the *Dictyostelium*-predicted proteins is 41% (Figure 1A). Homologues are also present in other organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arapidopsis thaliana* (Figure 1B). However, no homologues were found in any fungi. Interestingly, it is present in other protists such as pathogenic protozoa, suggesting a specific gene loss during fungi evolution. Furthermore, *Dictyostelium* Vmp1 is more similar to the vertebrate homologues than to other simpler organisms, including other protists. The hydropathicity profile between the *Dictyostelium* and human proteins also showed a high level of similarity, suggesting conservation in the predicted transmembrane domains (Figure 1C). The *Dictyostelium vmp1* gene was disrupted by homologous recombination using an optimized protocol based on in vitro transposition (Torija *et al*., 2006a,b). Figure 1D shows a scheme of the gene and the point of insertion of the blasticidin cassette. Two oligonucleotides surrounding the disruption were used to screen for homologous recombination by PCR as depicted in Figure 1D. Several independent mutants were obtained that showed the same phenotype, and one of them was chosen for further analysis. The lack of expression of *vmp1* mRNA in the disruptant strain suggests that the insertion generated a loss-of-function mutant (Figure 1E). *vmp1* disruption was also generated in other *Dictyostelium* strains (Table 1), and the phenotype was similar regardless of the strain background.

The major consequences of *vmp1* disruption are shown in Figure 2. Growth in association with bacteria was slightly affected as observed by the size of the clearing plaques (Figure 2A). These cells were also deficient in initiating development upon starvation (see also below). Cell growth in axenic media (HL5) was compromised in shaking culture. Cells grew slowly over the first 2 d in culture, and cell growth eventually stopped completely. However, when cells were set in Petri dishes, allowing them to attach to the plastic, their growth was very slow but sustained (data not shown). The possibility of a cytokinesis defect was studied by staining the cells with 4,6-diamidino-2-phenylindole (DAPI). No differences in the number of nuclei per cell were found in any growth condition (data not shown). Intriguingly, when mutant cells were directly taken from SM plates and incubated in water they rapidly rounded up and after few minutes a proportion of them began to burst. This can be recognized by the presence of remaining cell debris (Figure 2B). This aspect of the phenotype is more patently illustrated in Supplemental Movie 1. The same phenotype was observed in cells previously incubated overnight in HL5. *Dictyostelium* cells have a contractile vacuole (CV) system, which allows the cells to efficiently survive hypoosmotic conditions. The presence and the activity of these vacuoles that expel water outside the cell can be visualized under phase contrast microscopy. A high magnification of the cells (Figure 2B) showed that whereas wild type displayed abundance of clear vacuoles that eventually fused with plasma membrane, the mutant cells were round and had a flat appearance with no evident activity of the CV (Figure 2B).

These results suggested a defect in osmoregulation and more specifically in the activity of the CV. To confirm this hypothesis, we incubated wild-type and mutant cells in different concentrations of sorbitol to generate a wide range of osmotic pressure. Figure 2C shows how the morphology of mutant cells became less round as the sorbitol concentration increases. At 100 mM sorbitol the cells looked more normal in appearance but nevertheless they showed no clear vacuole activity as seen in wild type. Mutant cells were able to respond to hyperosmotic stress (400 mM sorbitol) by reducing their volume (also known as cringing) and they showed high refringence, as described for wild type cells (Kuwayama *et al*., 1996). These results suggested a specific defect of the mutant cells in coping with hypoosmotic conditions as a result of impaired CV activity.

Vmp1 Is Required for Contractile Vacuole Biogenesis

To characterize the function of the CV in vivo, we disrupted *vmp1* in a *Dictyostelium* strain expressing the protein Dajumin fused to GFP (see Table 1 for a complete list of strains used in this report). Dajumin is a protein specifically located in the CV (Gabriel *et al*., 1999). Figure 3A shows the expected pattern of fluorescence in wild type. However, GFP fluorescence was hardly detectable in the disruptant strain, confirming the absence of functional vacuoles as observed under phase contrast microscopy. For further confirmation, we used the CV-specific antibody Rh50 (Benghezal *et al*., 2001). Figure 3B shows the immunofluorescence staining in wild type with the expected pattern. However, in this case a weak

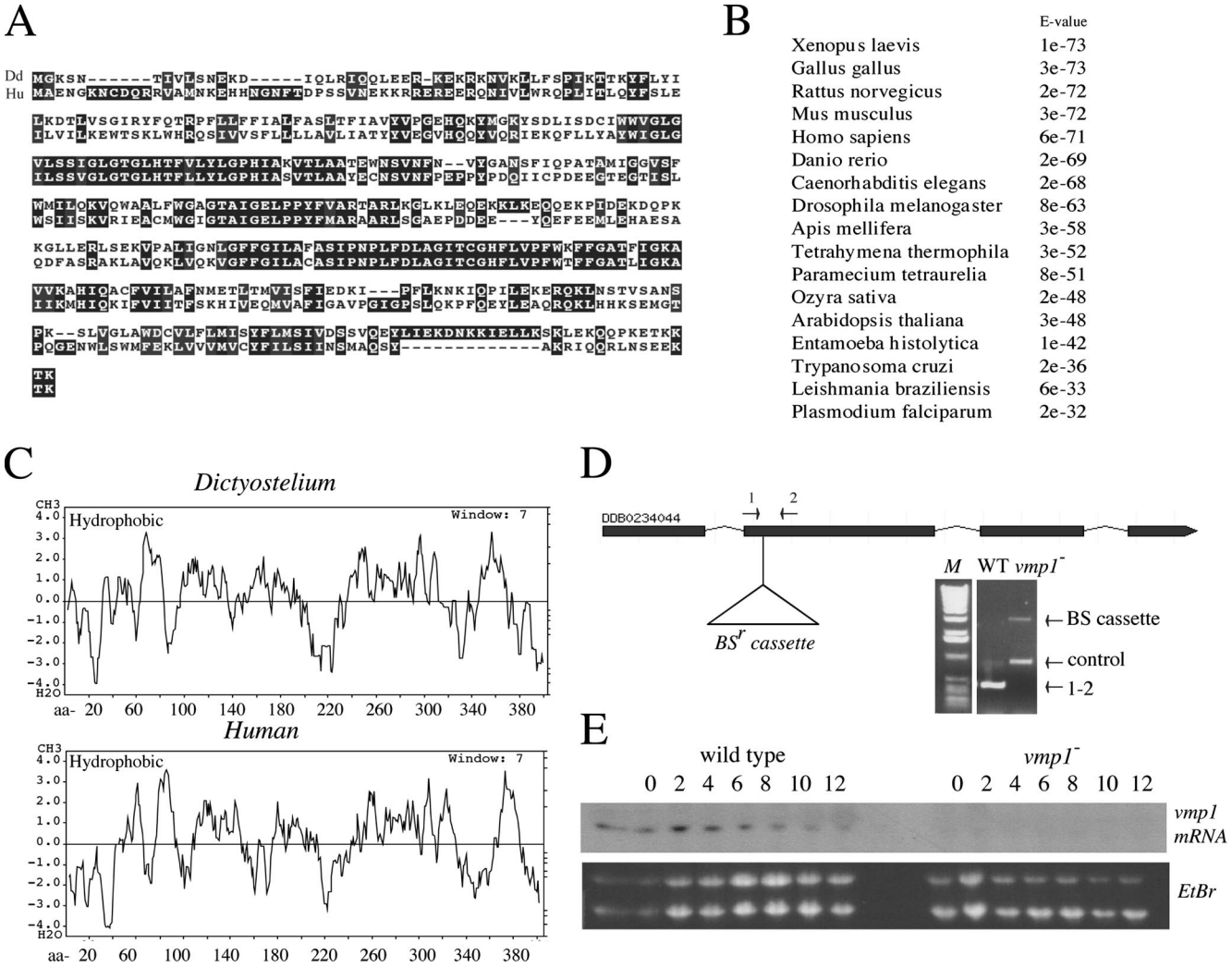

Figure 1. Disruption of the conserved gene *vmp1* in *Dictyostelium*. (A) The sequence of *Dictyostelium* Vmp1 predicted protein (DDB0234044) was aligned with the human homologue (CAG38552) by using the ClustalW program. The letters with black background correspond to identical residues and the gray background to similar residues. (B) The most similar proteins to the *Dictyostelium* Vmp1 are listed in order, from the highest identity to the lowest. (C) The hydropathicity plot of *Dictyostelium* and human Vmp1 predicted proteins are compared. The analysis was performed by the method of Kyte and Doolittle by using the online program at http://workbench.sdsc.edu/. (D) The structure of the *vmp1* gene is shown. Open boxes correspond to coding regions and the thin lines represent the introns. The insertion of the blasticidin cassette took place at the beginning of the second exon (amino acid 89) as indicated below. Disruption of the gene was assessed by PCR using oligonucleotides located at both sides of the insertion as indicated by the arrows. A pair of oligonucleotides from an unrelated locus was used as internal control of the PCR reaction. The panel below shows a typical analysis using DNA isolated from wild type (labeled as WT in the figure) and a disruptant strain (*vmp1*$^-$). The expected band (1–2) shifts to a higher band (BS-cassette) as a consequence of the insertion. M correspond to DNA size markers. (E) RNA isolated at different times of development was hybridized with a radioactive probe derived from the coding sequence of Vmp1. The band detected in wild type is absent in the disruptant strain.

signal was detected in the mutant, although the intensity of the staining and the size and number of vacuoles was greatly reduced (Figure 3B).

Dictyostelium cells contain many large vacuoles, including CVs and also vacuoles of the endocytic pathway, which are mainly devoted to nutrition in *Dictyostelium* (Maniak, 2003). Some of these vacuoles can be distinguished by electron microscopy. As seen in Figure 4, WT cells showed a high number of large electroluscent vesicles. Some of them contained spongy material that is believed to correspond to different degrees of digestion of the axenic media that has been internalized by macropinocytosis (Ryter and de Chastellier, 1977). Some of these vacuoles were completely electron transparent and probably correspond to contractile vacuoles (Ryter and de Chastellier, 1977). Mutant cells showed some striking differences with WT cells. First, there was a remarkable reduction in the number of electroluscent vacuoles, suggesting a defect not only in the biogenesis of the CV system but also in the endocytic pathway (aspect addressed below). Second, mutant cells showed an abnormal accumulation of electrodense vacuoles enclosing large granular and membranous material (Figure 4). The ratio of the number of electroluscent/electrodense vacuolar profiles ($\geq$200 nm) per μm^2 of cytoplasm was determined by morphometric analysis. This ratio in WT was 3.45 $\pm$ 0.98 (number of cells analyzed, n = 14), and it was significantly higher ($p \leq 0.0001$) than that obtained in the mutant, 0.43 $\pm$ 0.11 (number of cells analyzed, n = 14). These electrodense vesicular profiles are very similar to those described in macroautophagy mutants in *Dictyostelium* that are believed to be

Table 1. Strains used in this study

Strain name	Characteristics	Genotype	Parental strain	Reference
AX4	Wild-type strain			
AX2	Wild-type strain			
vmp1$^-$	Gene disrupted at aa-89	BS^r	AX4	This report
vmp1$^-$*/act15*::DdVmp1	Complemented strain by the expression of *Dictyostelium* Vmp1-GFP fusion protein	G418^r; BS^r	Vmp1$^-$	This report
vmp1$^-$*/act15*::rVmp1	Complemented strain by the expression of rat Vmp1-GFP fusion protein	G418^r; BS^r	Vmp1$^-$	This report
Vmp1-GFP	Complemented strain by single homologous recombination	G418^r; BS^r	Vmp1$^-$	This report
act15::ratVmp1	Expression of rat Vmp1 in wild type	G418^r	AX2	This report
Golvesin(C)-GFP	Marker for the Golgi apparatus	G418^r	AX2	Schneider *et al.* (2000)
Golvesin(C)-GFP/Vmp1$^-$	Marker for the Golgi apparatus in *vmp1*$^-$	G418^r; BS^r	Golvesin(C)-GFP	This report
Dajumin-GFP	Marker of the contractile vacuole	G418^r	AX2	Gabriel *et al.* (1999)
Dajumin-GFP/Vmp1$^-$	Marker of the contractile vacuole in *vmp1*$^-$	G418^r; BS^r	Dajumin-GFP	This report

nonmature autophagosomes containing nondigested cytoplasm and organelles (Otto *et al.*, 2003, 2004). This observation suggests a possible implication of Vmp1 in macroautophagy.

Vmp1 Is an Endoplasmic Reticulum Protein in Dictyostelium

We next cloned the entire *Dictyostelium vmp1* gene fused to GFP as a reporter for subcellular localization studies. The expression of the composite gene was driven by the constitutive actin 15 promoter. Mutant cells were transformed with the construct and the resulting strains fully complemented the phenotype in growth, development and osmoregulation (Figure 5, A and B). Complementation of the phenotype strongly suggests that the fused protein is functional. The complemented strain was used for confocal studies to determine the subcellular localization of the protein. The GFP fluorescence pattern as shown in Figure 5C, suggested that the protein was localized in internal membranes. We therefore used several markers of intracellular compartments for colocalization by immunofluorescence. Although a prominent defect in the mutant is related with the CV and the endosomal pathway (see below), Vmp1 did not colocalize with V-ATPase (VatA), a CV/endosomal protein (Clarke *et al.*, 2002), or with the endosomal marker P80 (Supplemental Figure 1). However, as shown in Figure 5D complete colocalization was observed with protein disulfide isomerase (PDI), a typical marker of the ER (Monnat *et al.*, 1997). Moreover, a strong GFP fluorescence labeled the nuclear envelope as can be seen in Figure 5D surrounding several nuclei stained with DAPI.

We next wanted to determine whether the expression of a mammalian Vmp1 in *Dictyostelium* was able to complement the mutant strain. The complete rat Vmp1 was cloned by RT-PCR and placed in frame with GFP in a similar construct as the one described for the *Dictyostelium* protein. This construct was transformed in wild type, and the mutant strains and stable transformants were isolated. As expected by the high conservation of the amino acid sequence, the mammalian protein was also localized in the endoplasmic reticulum in both strains (Supplemental Figure 2). Interestingly, growth in axenic media and development were almost completely recovered as well as the resistance to hypoosmotic conditions as described in Supplemental Figure 2. These results strongly suggest that the mammalian protein is functionally similar to the *Dictyostelium* protein.

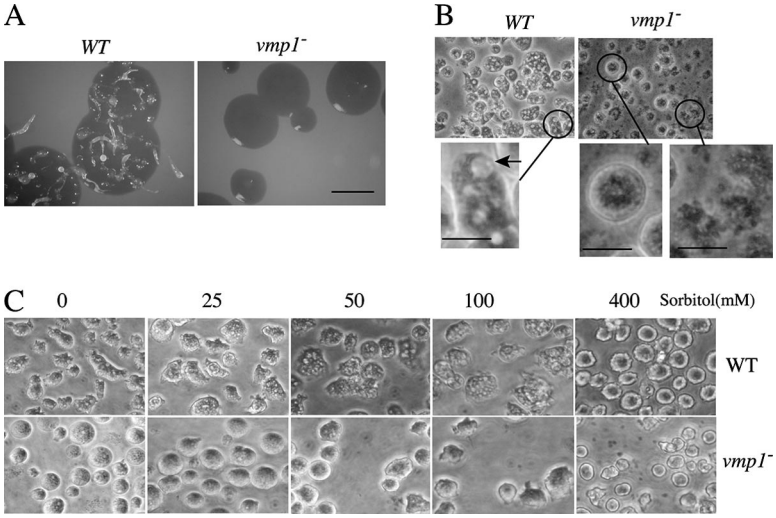

Figure 2. Vmp1 mutant cells are defective in growth, development and osmoregulation. (A) Wild type and *vmp1*$^-$ cells were cultivated in association with *Klebsiella aerogenes* in SM plates. The clearing plaques in the mutant are slightly smaller than those of the WT and do not show any sign of aggregation and development. Bar, 1 cm. (B) Cells were taken from the growing zones of *Klebsiella*-SM plates and incubated in water. After ~30 min, phase contrast photographs were taken with a Nikon Eclipse microscope. Wild type showed extensive vacuolization. A magnified image shows a contractile vacuole before discharging water outside the cell. The mutant cells were round and had a flat appearance with few vacuoles. Some of the mutant cells showed evidence of cell lysis. Magnified images show a mutant cell and cell debris. Bar, 10 μm. (C) Wild-type and mutant cells were incubated at different concentrations of sorbitol to study their response to osmotic pressure. Cell rupture and morphology were gradually recovered in the mutant as the osmotic pressure increased. Bar, 10 μm.

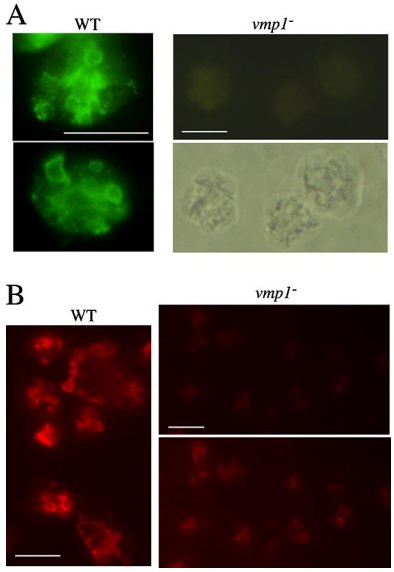

Figure 3. Analysis of the contractile vacuole activity. (A) *vmp1* was disrupted by homologous recombination in a strain expressing Dajumin fused to GFP, a marker of the CV network in *Dictyostelium*. WT and mutant cells were fixed and observed in a Nikon fluorescence microscope. Two representative cells of WT showed the expected pattern. However, fluorescence was barely detected in the mutant (top). Bottom, bright field image of the cells. (B) Immunofluorescence using Rh50, a CV-specific antibody coupled to Alexa 546, was performed in wild-type and mutant cells. A field containing several cells is shown for WT and the mutant. Although some staining was detected in the mutant, the intensity and the size of the vacuoles were reduced. The mutant top panel shows a photograph taken under the same intensity and contrast than the one shown for WT at the left. The bottom panel is the same picture with increased contrast to reveal the weak staining. Bars, 10 μm.

Vmp1 Is Required for ER Integrity and Membrane Traffic-dependent Processes

Although Vmp1 is present in the endoplasmic reticulum, we have shown evidence of defects in the CV. Because the ER is the starting point of the secretory pathway, we hypothesized that a primary defect in the structure of the ER, generated by the loss of Vmp1, could affect the biogenesis and the function of other organelles as a result of defective membrane traffic. To test this hypothesis, we analyzed the structure of the ER and the Golgi apparatus, endocytic trafficking, and the kinetics of protein secretion in the *vmp1*$^{-}$ mutants. We found acute alterations in most of these processes, which are summarized in Figure 6.

The ER in *Dictyostelium*, as observed by immunofluorescence microscopy with the PDI marker, has a typical tubulovesicular structure in WT (Figure 6A). Remarkably, it showed a fragmented appearance in the mutant suggesting a role for Vmp1 in the maintenance of its normal membrane structure. To visualize the Golgi apparatus in the mutant cells, Vmp1 was disrupted in a strain expressing Golvesin fused to GFP, which specifically labels this organelle (Schneider *et al.*, 2000). As displayed in Figure 6B, whereas wild-type cells usually showed fluorescence in a single and restricted area close to the nucleus, most of the mutant cells showed dispersed and fragmented areas of fluorescence.

The endocytic pathway in *Dictyostelium* is primarily dedicated to nutrition, and it depends, as in the rest of eukaryotes, on membrane traffic processes. We have studied the time course of endocytosis and exocytosis as a measure of the functional activity of endocytic organelles. Macropinocytosis as determined by the internalization of fluorescent markers was dramatically affected in the mutant (Figure 6C). Moreover, the staining of F-actin with phalloidin-rhodamine in wild-type and mutant cells showed a clear difference in the presence of crown-like structures (data not shown). Crowns are actin structures located in macropinosomes marking the early stages of macropinocytosis (Hacker *et al.*, 1997). There were almost no such structures in the mutant (data not shown). Exocytosis was difficult to evaluate because the cells accumulated much less fluorescent marker than the wild type shown in Figure 6D. Even so, the slope of the secretion graph at any point was also reduced in the mutant. As anticipated by the ability of the mutant cells to grow in association with bacteria, phagocytosis of fluores-

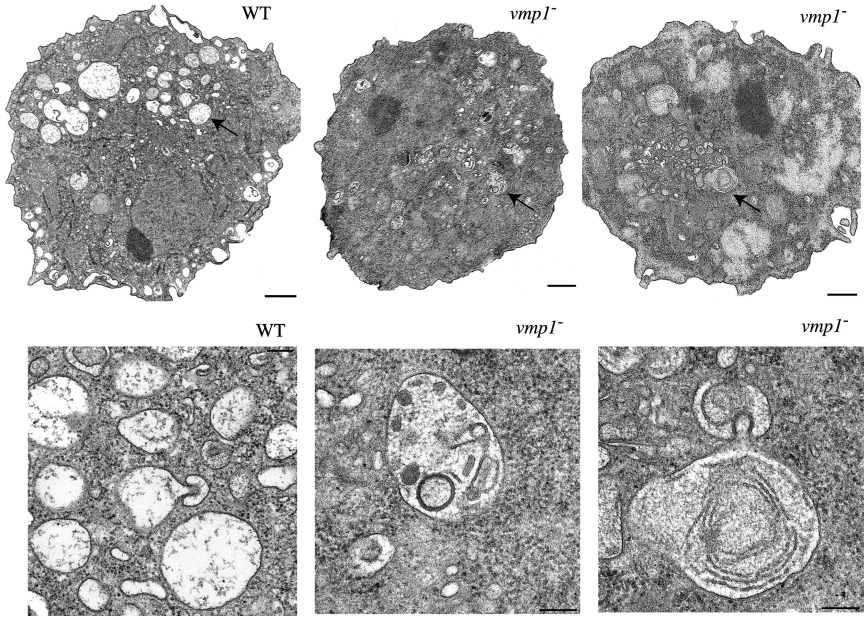

Figure 4. Electronic microscopy of WT and mutant cells. The arrows aim to the magnified area that is shown below. Mutant cells show accumulation of electrodense vacuoles enclosing large granular and membranous material. Bars, 1 μm (top); 200 nm (bottom).

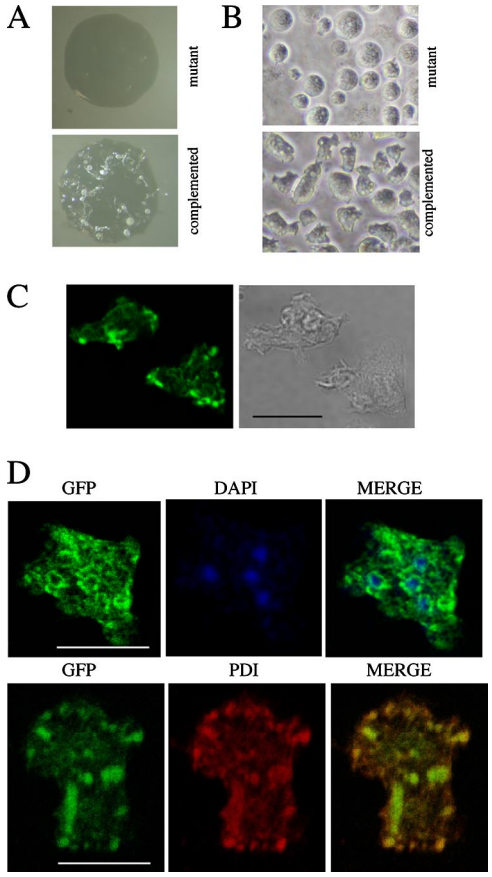

Figure 5. Vmp1 is an endoplasmic reticulum protein in *Dictyostelium*. *Dictyostelium vmp1* gene was fused to GFP and transformed into the *vmp1*$^-$ mutant. The transformant strain showed a WT phenotype with respect to development, osmoregulation and growth. (A) Mutant and complemented strains were grown in SM plates in association with *K. aerogenes*. (B) The strains were incubated in water for 30 min, and the complemented strain showed a normal response to hypoosmotic conditions as can be seen by the irregular shape of the cells and the presence of vacuoles. (C) Complemented cells were fixed and the fused Vmp1-GFP protein was visualized by confocal microscopy. Left, GFP fluorescence Right, bright field of the same cells. Bar, 10 μm. (D) The complemented strain expressing the fusion Vmp1-GFP protein was stained with DAPI (top) or treated for immunodetection of the endoplasmic reticulum marker PDI (bottom). The merged images show the presence of GFP fluorescence in the nuclear envelopes and colocalization with PDI. Bars, 10 μm.

cent beads was observed in the mutant, although at lower rate than in WT (Figure 6E).

Protein secretion is a process dependent on membrane traffic. Therefore, we wanted to determine a possible role of Vmp1 in the kinetics of secretion of AprA, a protein secreted by a conventional mechanism (ER–Golgi transit) that regulates *Dictyostelium* growth (Brock and Gomer, 2005). Wild-type and mutant cells were set in fresh HL5 media, and aliquots were taken at the indicated times. The amount of AprA secreted to the media was analyzed by Western blot (Figure 6F). A rapid accumulation of the protein in the extracellular media was observed in wild type. However, very little protein was detected in the mutant media suggesting a defect in protein secretion. The presence of intracellular AprA was also determined as a control.

Vmp1 Is Necessary for the Transition from Growth to Development

As shown in Figures 2 and 7A, *vmp1*$^-$ mutant cells were unable to aggregate in association with bacteria or on nitrocellulose filters soaked with PDF. We have previously determined that mutant cells, in contrast to their response in water, remained viable in PDF buffer and showed no signs of cell lysis (data not shown). Nevertheless, no aggregation of mutant cells was observed when PDF was supplemented with sorbitol (50 and 100 mM), suggesting that the deficiency in initiating development was not due to their osmosensitivity (data not shown). What aspects of the phenotype could account for such block in development? It is well known that the transition from growth to development in *Dictyostelium* is dependent on the activation of a specific gene expression pattern involving many genes that are regulated by extracellular signals induced by starvation (Kessin, 2001). Among them there are genes coding for the synthesis and relay of cAMP signaling that are essential for aggregation. We studied by Northern blot the expression pattern during early development of some of these markers. As shown in Figure 7B, the level of expression of the cAMP receptor Car1 and the adenylyl cyclase ACA were barely detected in the mutant. The absence of expression of any of these genes would be sufficient to account for the lack of aggregation as seen in the mutant. Discoidin I, in contrast, is a developmentally regulated lectin whose expression depends on cell density. The level of expression during early development was also strongly reduced in the mutant. As a control of constitutive expression, *patA*, a gene coded for a P-type ATPase, was used (Moniakis *et al*., 1995).

In *Dictyostelium*, the expression of these early developmental markers and discoidin are dependent on several secreted proteins during growth and starvation such as prestarvation response factor and conditioned medium factor (Clarke and Gomer, 1995) among others. Therefore, the observed impairment in the starvation response could be due to an abnormal cell response to these extracellular proteins, or to the absence of those signals as a result of abnormal protein secretion, as observed for AprA during growth, or more likely the combination of these two possibilities. Mixing experiments of mutant cells with different proportions of WT were unable to rescue mutant development, suggesting a cell autonomous defect (data not shown). Nevertheless, we undertook a proteomic approach to identify possible differences in the pattern of secreted proteins. Conditioned media was obtained by incubation of cells in starvation during 7 h. The media were then washed free of cells by centrifugation, and the secreted proteins were analyzed by SDS-PAGE electrophoresis. A direct comparison of the protein pattern between wild type and the mutant is shown in Figure 8. Most of the protein bands were present in both media with small differences in abundance. However, some striking differences were observed. Some protein bands were present in wild type but absent in the mutant and more interestingly there were bands present in the mutant that were barely detectable in wild type. This result might indicate a more complex scenario than just the lack of certain protein complement in the mutant suggesting an aberrant regulation of protein secretion.

We next tried to identify the differential protein bands by MALDI-MS/MS. Three different proteins were successfully identified (Figure 8). α-Mannosidase precursor (DDB0201569) and a cysteine proteinase (DDB0219654) were present in WT conditioned media but almost absent in the mutant. These two proteins are secreted by conventional mechanisms (ER/

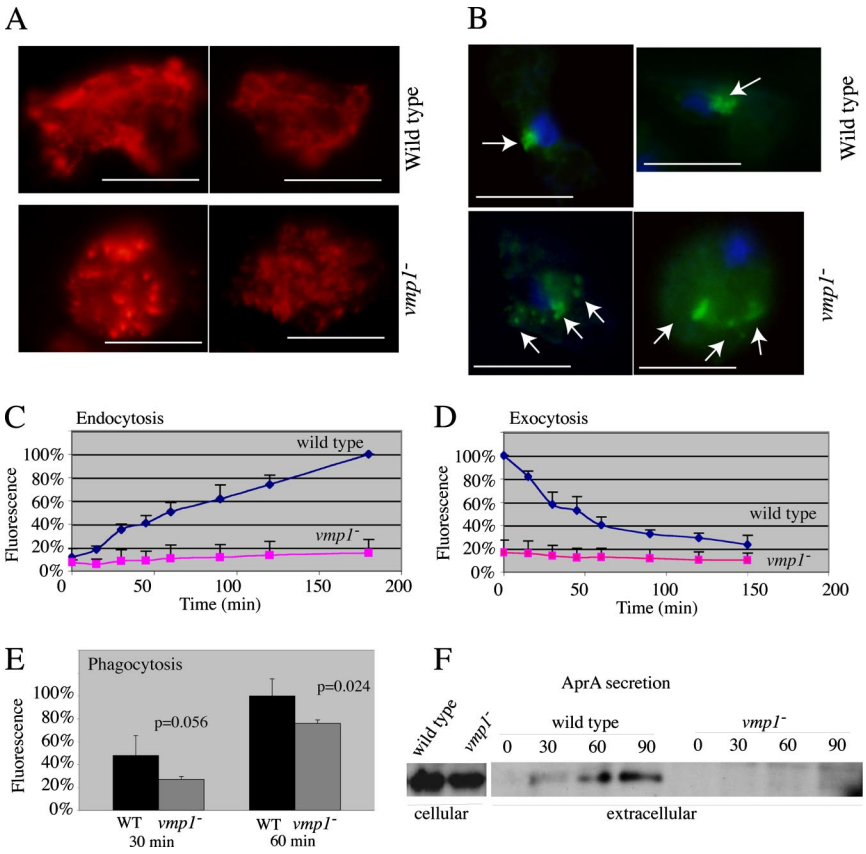

Figure 6. Characterization of defects in other membrane-traffic dependent processes. (A) Antibody label of protein disulfide isomerase (PDI), a marker of the endoplasmic reticulum. Photographs were taken in a fluorescence microscope and two representative cells of each strain are shown. Mutant cells showed fragmented endoplasmic reticulum. Bar, 10 μm. (B) *vmp1* was disrupted in a strain expressing Golvesin-GFP, a marker of the Golgi apparatus. The morphology of the Golgi apparatus was observed by fluorescence microscopy in the mutant and the parental strain. The Golgi apparatus in the mutant seemed disorganized. (C) Endocytosis assay. Wild-type and mutant cells were incubated in the presence of a soluble fluorescent marker. At the times indicated, the internal cellular fluorescence was determined. The mean of three independent experiments and the SD is shown. (D) Exocytosis assay. Cells were first preloaded with the marker and the decrease in internal fluorescence was measured at the times indicated. The mean of three independent experiments, and the SD is shown. (E) Phagocytosis assay. WT and mutant cells were exposed for the indicated times to fluorescent beads. Fluorescence was expressed as arbitrary units. The mean of four independent experiments is shown and the significance of differences is indicated by the p value. Bars show the SD. (F) Protein secretion assay. Wild-type and mutant cells were incubated in HL5 for the indicated times and the secreted AprA was analyzed in the media by Western blot using a specific antibody. The cellular extract was also analyzed as a control.

Golgi), reinforcing the data obtained by AprA (Pannel *et al*., 1982; Wood and Kaplan, 1985). Interestingly, a band overrepresented in the mutant media was identified as 70-kDa hear shock protein (Hsp70) (DDB0219654), a chaperone that has been described to play an additional role as an extracellular protein secreted by nonconventional mechanisms (Mambula *et al*., 2007; Multhoff, 2007). Although a comprehensive proteomic analysis would be necessary to fully characterize the defects in mutant conditioned media, our findings suggest that Vmp1 is also required for normal protein secretion during starvation.

DISCUSSION

Analysis of Vmp1 in Dictyostelium Reveals a Complex Function for This New Protein

Vmp1 is a conserved eukaryotic protein that seems to be lost in the fungi lineage during evolution. Consequently, *Dictyostelium* is one of the simplest genetically tractable model systems to address its function. This is the first loss-of-function mutant described for this gene in an experimental system, and Figure 9 shows a summary of the defects observed in the mutant in the context of the secretory pathway.

Our results show that Vmp1 is an ER protein required to maintain the structure of this organelle. Despite its location, it is involved in a wide range of membrane traffic-dependent processes such as organellar biogenesis and protein secretion. The morphology of the ER itself, the Golgi apparatus and the CV are compromised in the mutant as well as the maturation of autophagosomes and the function of the endocytic pathway. Protein secretion during growth and starvation is also dependent on Vmp1. It is conceivable that a primary defect in the ER, which is the starting point of the secretory pathway, could have an impact on other processes, which are directly dependent on the correct trafficking of protein and membrane components. This is not without precedent. For example, defective ER-resident proteins CLN6 and CLN8 are responsible for lysosomal dysfunctions (Kyttala *et al*., 2006). Secretion of extracellular matrix is impaired when CopII coat component Sec 23a is mutated as a result of defective transport from the ER (Lang *et al*., 2006).

We have found that some of these defects such as the disorganization of ER and Golgi are gradually aggravated over time as the cells remain in HL5 axenic culture. Cells in these conditions are likely to be starved due to the observed defects in nutrient uptake, suggesting that starvation might aggravate those phenotypes. However, other defects such as osmosensitivity and the onset of development do not seem to depend on the growth conditions. Cells taken directly from SM-plates, where they are feeding on bacteria to almost normal rates did not survive hypoosmotic conditions and were unable to initiate multicellular development.

Vmp1 Subcellular Localization and Its Functional Implications

Several lines of evidence suggest that Vmp1 is an ER-resident protein in *Dictyostelium*. We have shown that the fused protein Vmp1-GFP is localized in the endoplasmic reticulum and no colocalization has been observed with CV or endosomal markers. The fused protein showed full complementation of the phenotype when transformed in the mutant strain, suggesting that the protein must be in a functional conformation and also localizes to in the correct cellular compartment. Moreover, a knockin strain was obtained by

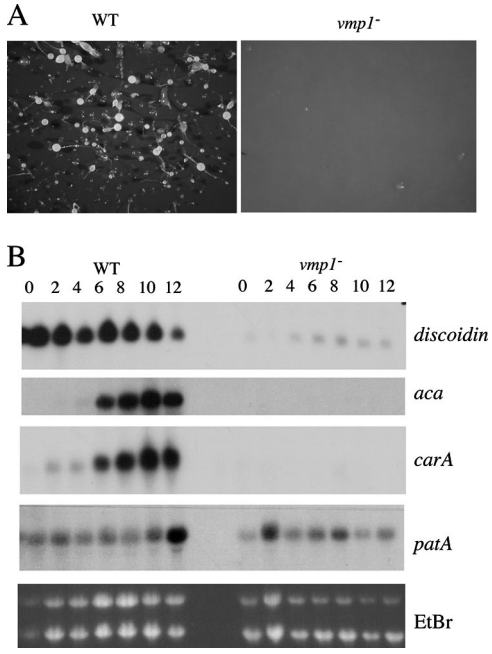

Figure 7. Developmental gene expression in *vmp1*$^{-}$ mutant. (A) WT and mutant cells were deposited in nitrocellulose filters for development and photographs of a representative experiment were taken after 30 h. No aggregation was observed in the mutant. (B) RNA was isolated form cells developed on nitrocellulose filters for the indicated times, transferred to nylon membranes and hybridized to radioactive probes. The same blot was stripped and hybridized several times. The expression of the lectin Discoidin, the adenylyl cyclase ACA, and the cAMP receptor Car 1 was greatly reduced. The expression of P-type ATPase (PatA), which is not developmentally regulated, was used as a control. The staining of ribosomal RNAs by ethidium bromide is shown at the bottom.

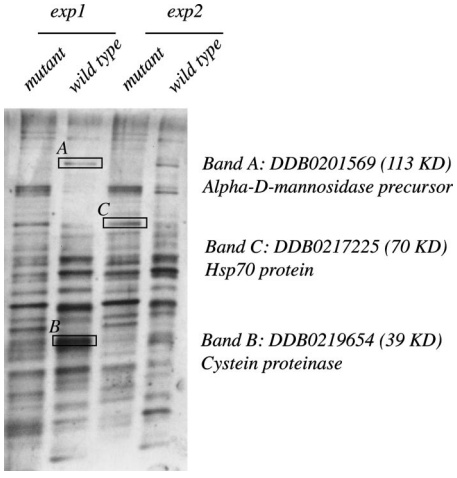

Figure 8. Conditioned medium and protein identification by MALDI-MS/MS. Wild-type and mutant cells were washed free of nutrients and resuspended in PDF for 4 h in shaking culture. Cells were then sedimented by centrifugation and the conditional media analyzed by SDS-PAGE. The gel was silver stained, and the protein pattern was compared. Two independent experiments are shown. Differential bands were cut and analyzed by MALDI-MS/MS. Three bands were successfully identified with a Mascot score of 1.1E-6 (band A), 5.6E-10 (band B), and 7.0E-6 (band C).

single crossover (Charette *et al*., 2006), in which a unique copy of the fused protein was under the control of the endogenous promoter (Supplemental Figure 3). This strain is expected to give rise to a level of expression that is similar to wild-type cells. In fact, the intensity of fluorescence was lower than that obtained in the overexpressor strains (data not shown). Even then, the localization was found in the ER as determined by colocalization with PDI (data not shown).

The amino acid sequence of *Dictyostelium* Vmp1 showed a conserved KKXX-like ER-membrane retention signal at the C terminus as detected by the PSORT program (Nakai and Horton, 1999), resulting in a K-NN prediction 66,7% for ER, which is confirmed by our results. In this connection, an ER localization for Vmp1 has also been described in *Drosophila* (Bard *et al*., 2006) and also in a proteomic study in *Arabidopsis* (Dunkley *et al*., 2006).

Regarding cell localization, the scenario in mammalian cells seems to be more complex. Vmp-1 has been reported to be localized in different subcellular compartments and these results suggest that it may play different roles. The first report by Dusetti *et al*., 2002 localized Vmp1 as a fused protein with GFP in vacuoles, the area of the ER, and the Golgi apparatus. Unfortunately, colocalization analysis with cell markers was not performed in this study to determine the precise localization. The expression of this Vmp1-GFP–fused protein induced vacuolization and death in Cos7 cells (Dusetti *et al*., 2002). Interestingly, our results have shown that a mammalian Vmp1 expressed in *Dictyostelium* also has an ER localization. Remarkably, this construct also complemented the mutant phenotype, suggesting correct subcellular targeting and functional conservation of the protein.

A second report by the same group several years later further defined the vacuolization induced by Vmp1 expression as autophagy in mammalian cells (HeLa293T and NIH3T3) (Ropolo *et al*., 2007). Autophagy is a degradation process of cytoplasmic cellular components that is essential as a survival mechanism during starvation (Klionsky and Emr, 2000). Vmp1 colocalized with LC3, a marker of the autophagosomes and induces autophagosome formation in pancreas acinar cells in transgenic mice (Ropolo *et al*., 2007). The endogenous expression of Vmp1 in HeLa cells was very low and only detectable under conditions inducing autophagome formation such as starvation. In this case, Vmp1 is detected in punctuate structures by immunofluorescence consistent with the autophagosome vesicle formation. It is possible as stated by the authors that a low nondetected basal Vmp1 expression could be related with other physiological processes (Ropolo *et al*., 2007).

Interestingly, a recent report showed that Vmp1 was located in the plasma membrane playing a role in cell–cell contact (Sauermann *et al*., 2008). We have never found *Dictyostelium* Vmp1 in the plasma membrane at the vegetative stage. We also checked the localization pattern during development (finger stage) to determine whether some localization was found in cell–cell contacts. However, the protein remained exclusively at the ER (data not shown).

Together, these results suggest that Vmp1 might be located in the ER in lower organisms, including the social amoebae, insects, and plants, in which it could play a basic role in membrane traffic as we have shown in *Dictyostelium*. In mammalian cells, Vmp-1 may play more diverse roles in different organelles including autophagosome vesicles and the plasma membrane. Whether Vmp1 has a specific role in the ER of mammalian cells as suggested by our results in *Dictyostelium* remains to be investigated.

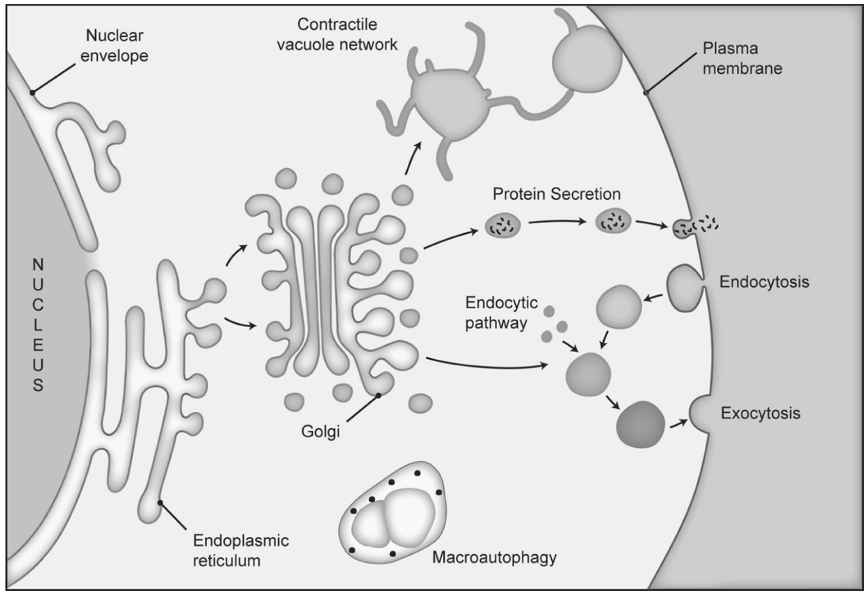

Figure 9. Summary of Vmp1-dependent processes. The amino acid sequence of the encoded protein contains several putative transmembrane domains suggesting that Vmp1 is an integral membrane protein located in the ER. Despite its localization, the loss of this gene in *Dictyostelium* leads to defects in several membrane traffic-dependent processes, including the biogenesis of the CV, the structure of the Golgi apparatus, and the pattern of macroautophagy vacuoles. In contrast, the endocytic pathway, which in *Dictyostelium* is essential for nutrition, is also affected. Endocytosis and exocytosis is severely impaired in the mutant. Protein secretion during growth and probably also during starvation is also defective.

Vmp1 Is Required for Osmoregulation, an Essential Process in Soil Microorganisms

The survival in hypoosmotic conditions is essential for single-celled organisms. These organisms, including *Dictyostelium*, have developed a complex network of vesicles and tubules known as the CV. If CV function is compromised as a result of a general defect in membrane trafficking, the expected phenotype would be dramatic. The cells would not be able to cope with water influx and may burst as a consequence of cell swelling. This is precisely what occurs in the absence of Vmp1 in *Dictyostelium*. A similar phenotype have been reported for a *Dictyostelium* mutant in the AP-1 clathrin-adaptor (Lefkir *et al*., 2003). AP-1 complex participates in vesicle transport from the *trans*-Golgi network to endocytic compartments. This mutant showed a severe growth defect and delayed development and most importantly the biogenesis of the contractile vacuole was compromised. This is another example of a defect in organelle biogenesis as a consequence of defective membrane traffic.

Our results suggest that Vmp1 mutant cells contain very few contractile vacuoles as determine by transmission electron microscopy (TEM) and the use of CV markers. These markers (mainly Rh50) showed a weak staining, labeling few vacuoles in the mutant. This staining did not colocalize with ER or endosomes (data not shown). The reduced levels of these proteins might suggest that the components of the CV system are being degraded or synthesized in lower amounts because they do not seem to be accumulated in other organelles of the secretory pathway.

Mammalian cells lack the contractile vacuole system as they live in an osmotically controlled environment. Therefore, no such behavior is expected to occur by the loss of mammalian Vmp1.

A Possible Role of Vmp1 in Macroautophagy

It has been described that inhibition of Vmp1 expression by small interfering RNA blocks autophagosome formation in mammalian cells (Ropolo *et al*., 2007). Our results in *vmp1*$^-$ mutant in *Dictyostelium* might also support a role in autophagy. TEM images of mutant cells showed the accumulation of autophagic features similar to those described in macroautophagy mutants (Otto *et al*., 2003, 2004). Macroautophagy is induced by starvation and therefore it is probably activated in *vmp1*$^-$ mutant cells in axenic culture as a result of the observed defects in nutrient uptake. Thus, we cannot rule out the possibility that the observed accumulation of vacuoles with unprocessed cellular material might be due to an overactivation of autophagy in the mutant. Other possible explanation is a defect in autophagosome maturation. Such maturation involves dynamic membrane rearrangements and the fusion with lysosomal vesicles that makes the process dependent on the secretory pathway (Mizushima *et al*., 2002). Moreover, the ER itself might, in some cases, be the membrane source for autophagosome formation (Bernales *et al*., 2007; Yorimitsu and Klionsky, 2007). Nevertheless, *Dictyostelium* macroautophagy mutants do not show growth defects or osmotic sensitivity (Otto *et al*., 2003, 2004), suggesting that macroautophagy dysfunction does not account for the overall phenotype of *vmp1*$^-$ mutant. The precise role of Vmp1 in *Dictyostelium* macroautophagy will require further investigation.

Protein Secretion during Growth and Development Depends on Vmp1

We have shown that protein secretion during growth and development is affected by the loss of Vmp1. AprA is an autocrine proliferation repressor in *Dictyostelium*; therefore, a lower level of secretion of this specific factor would induce growth as described previously (Brock and Gomer, 2005). The *vmp1*$^-$ mutant, however, showed a severe growth defect in axenic media. This implies that AprA is not the only factor that is likely to be affected by a general dysfunction in protein secretion. Other unidentified growth-inducing factors may be absent and/or other additional defects may account for the lack of growth in shaking culture.

It is well known that secretion of autocrine factors is necessary for the transition from growth to development in *Dictyostelium* and that this transition is dependent on the onset of a specific gene expression program. We have shown that the expression of representative genes of this program is severely affected and the pattern of protein secretion is also different between wild type and the mutant. If the observed block in development is merely a consequence of the absence of these autocrine factors, we would expect a noncell

autonomous behavior in mixing experiments. However, when mutant cells were mixed with wild-type, mutant cells remained excluded from the aggregates when development was initiated (data not shown). This additional cell-autonomous defect is not surprising if taking into account the severity of the phenotype, which is not only restricted to protein secretion.

The pattern of proteins present in conditioned medium from wild type and the mutant Vmp1 is significantly different. This could be due to differences in protein secretion as well as differences in the expression levels of certain genes because the mutant response to starvation is impaired. Despite our efforts we have been able to identify only few of the differential protein bands. The secreted proteins enriched in the wild-type medium are representative of ER/Golgi-dependent secretory pathway. However, others such as Hsp70 are secreted by nonconventional secretion mechanisms (Nickel, 2005). In this case, Hsp70 was described to be internalized into endolysosmal vesicles by the activity of an ATP-binding cassette transporter, and these vesicles secrete their content by fusing with the plasma membrane (Mambula *et al*., 2007). The high levels of Hsp70 in the mutant supernatant might be related to the activation of stress mechanisms. However, RT-PCR analysis of the hsp70 mRNA levels did not show an induction of the expression of the gene in the mutant (data not shown).

In conclusion, the analysis of Vmp1 null-mutant in *Dictyostelium* has brought us a more comprehensive view of the intricate roles played by this new gene. Our results confirm some data obtained in other experimental systems with regard to the role of Vmp-1 in membrane traffic and protein secretion, but also reveal new functions of Vmp-1, including an essential role in organelle biogenesis and multicellular development.

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CAPÍTULO 2: VACUOLE MEMBRANE PROTEIN 1, AUTOPHAGY AND MUCH MORE

Este segundo artículo sobre Vmp1 es un artículo adenda al capítulo 1 donde no se muestran nuevos resultados, pero sí se discute de una manera más amplia, la posible función y localización de Vmp1 en base a la bibliografía existente.

Los datos presentes en el capítulo 2 muestran que Vmp1 es una proteína de retículo endoplásmico que podría regular de algún modo multitud de procesos con origen en este orgánulo, sin embargo la bibliografía que recientemente se había publicado en líneas celulares de mamífero abogaba por una localización en autofagosomas y una función exclusivamente focalizada en autofagia. Incluso había sido localizado en membrana plasmática con una posible relación con cáncer e invasividad tumoral.

Las diferentes localizaciones y funciones parecen depender del tipo celular o el abordaje realizado.

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Article Addendum

Vacuole membrane protein 1, autophagy and much more

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Key words: vacuole membrane protein 1, organelle biogenesis, autophagy, protein secretion, osmoregulation, *Dictyostelium*, endoplasmic reticulum

Vacuole membrane protein 1 (Vmp1) is a putative transmembrane protein that has been associated with different functions including autophagy, cell adhesion, and membrane traffic. Highly similar proteins are present in lower eukaryotes and plants although a homologue is absent in the fungi lineage. We have recently described the first loss-of-function mutation for a Vmp1 homologue in a model system, *Dictyostelium discoideum*. Our results give a more comprehensive view of the intricate roles played by this new gene. *Dictyostelium* Vmp1 is an endoplasmic reticulum-resident protein. Cells deficient in Vmp1 display pleiotropic defects in the context of the secretory pathway such as organelle biogenesis, the endocytic pathway, and protein secretion. The biogenesis of the contractile vacuole, an organelle necessary to survive under hypoosmotic conditions, is compromised as well as the structure of the endoplasmic reticulum and the Golgi apparatus. Transmission electron microscopy also shows abnormal accumulation of aberrant double-membrane vesicles, suggesting a defect in autophagosome biogenesis or maturation. The expression of a mammalian Vmp1 in the *Dictyostelium* mutant complements the phenotype suggesting a functional conservation during evolution. We are taking the first steps in understanding the function of this fascinating protein and recent studies have brought us more questions than answers about its basic function and its role in human pathology.

Vmp1, a Membrane Protein of Unknown Function

Vacuole membrane protein 1 (Vmp1) was first described as a gene highly expressed in the pancreas during acute pancreatitis.[1,2] Overexpression of this protein in cell culture induces intracellular vacuolization and cell death.[1,3] These vacuoles were later characterized as autophagosomes, implicating Vmp1 as a novel autophagy protein.[4,5] In a functional genomic RNA-interference screening in Drosophila, Vmp1 (known as TANGO-5) is identified as a protein required for conventional protein secretion and Golgi organization.[6] Another surprise comes from a study in kidney cancer cells where Vmp1 is localized in the plasma membrane and required in adhesion.[7] The study of the function of this new gene is complicated by the fact that no conserved functional motifs are present in its encoded sequence and no homologous genes exist in the fungal models, where most of the autophagy proteins have been studied. The amino acid sequence only indicates the presence of several putative transmembrane regions and an endoplasmic reticulum (ER) retention signal.

Dictyostelium discoideum is a eukaryotic microorganism used as a model system for several cell biology processes including autophagy and non-apoptotic cell death.[8-12] Functional genomics in yeast has proven very valuable to understand the basic function of new genes that later were found to be involved in human diseases. However, there are many genes present in the human genome that are absent in the yeast models. Fortunately, the *Dictyostelium* genome contains many of those genes, being the simplest model system that can be used to address their function.[13,14] Some of these genes do not allow a prediction of their function due to the lack of characterized functional motifs. Vmp1, as explained above, complies with all these characteristics and was selected from a collection of mutants generated in *Dictyostelium* by our group.[13,15] This is the first loss-of-function mutation of Vmp1 analyzed in a model system and our studies reveal unexpected functions for this new protein beyond autophagy.

Subcellular Localization of Vmp1 and its Functional Conservation

The coding sequence of *Dictyostelium* vmp1 (DDB0234044) is highly similar to other homologous genes in other species. Interestingly, it is absent in the fungi lineage but it is present in other protists, plants, and metazoa, suggesting that this gene was lost during fungi evolution (Fig.1). Remarkably, *Dictyostelium* Vmp1 is even more closely related to metazoan genes than to those of other simpler eukaryotes or plants. In order to ascertain the functional conservation of this family, we expressed a mammalian Vmp1 protein in the *Dictyostelium* mutant. The expressed protein is able to complement the phenotype of the mutant and localizes in the same cellular compartment as the *Dictyostelium* protein, suggesting a functional conservation during evolution. Both proteins localize in the endoplasmic reticulum (ER) and no colocalization is observed with endosomal or contractile vacuole markers. There is some evidence of similar localization in plants and Drosophila.[6,16] However, Vmp1

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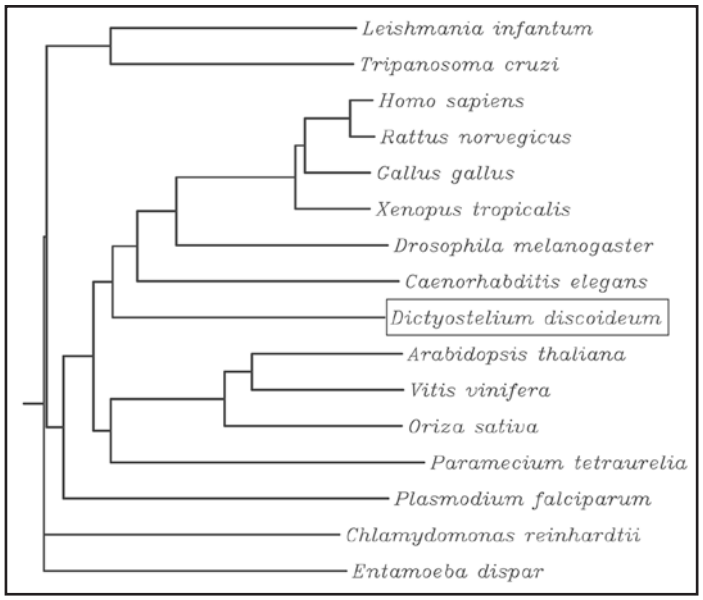

Figure 1. Phylogenetic relationships among Vmp1 proteins from different species. The phylogenetic tree was constructed using Dawgram, a PHILIP rooted phylogenetic tree available at Biology WorkBench (http://workbench.sdsc.edu). The sequences used were: *Dictyostelium discoideum*, XP_638348; *Xenopus tropicalis*, NP_001007877; *Gallus gallus*, XP_415880; *Rattus norvegicus*, NP_620194; *Homo sapiens*, NP_112200; *Caenorhabditis elegans*, NP_499688; *Drosophila melanogaster*, NP_727444; *Vitis vinifera*, CAO71145; *Paramecium tetraurelia*, XP_001432377; *Oryza sativa*, NP_001044428; *Arabidopsis thaliana*, AAM60984; *Entamoeba dispar*, XP_001735614; *Chlamydomonas reinhardtii*, XP_001696213; *Trypanosoma cruzi*, XP_815596; *Plasmodium falciparum*, XP_001348888; *Leishmania infantum*, XP_001470108. The highly divergent *Entamoeba* sequence was used as an outgroup.

Table 1 **Summary of Vmp1-dependent phenotypes in Dictyostelium**

Biological process	Defect and phenotype
Osmoregulation	Contractile Vacuole biogenesis Cell lysis in hypoosmotic conditions
Protein secretion	AprA secretion during growth
Organelle organization	Fragmentation of the ER Fragmentation of the Golgi apparatus
Autophagy	Aberrant autophagosome formation
Endocytic pathway	Impaired macropinocytosis (lack of axenic growth) Impaired exocytosis
Multicellular development	Lack of aggregation Impaired developmental gene expression

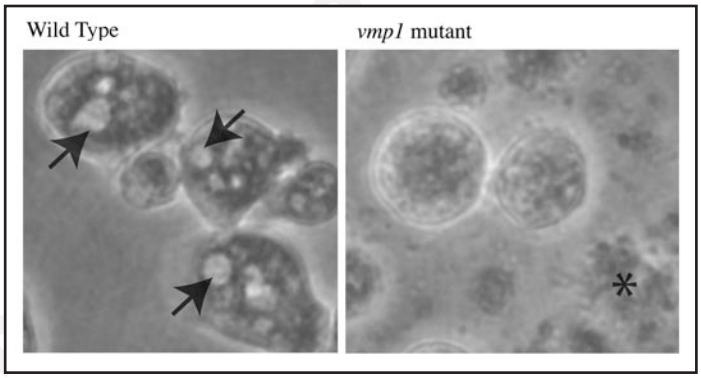

Figure 2. Cell lysis in vmp1 mutant. After a short exposure to water, wild type cells maintain their viability by expelling water through the CV (arrows). Mutant cells, in contrast, were round and showed no sign of CV activity. A large proportion of them lysed rapidly (asterisk) without a large increase in cell volume, suggesting the possibility of additional defects in the stability of the plasma membrane.

localization in mammalian cells is controversial and the protein has been described to be located in autophagosomes[5] and the plasma membrane.[7] We have never observed *Dictyostelium* Vmp1 in the plasma membrane but a possible localization in autophagosomes needs to be determined.

Vmp1 Disruption in *Dictyostelium*, Autophagy and Much More

Disruption of Vmp1 in *Dictyostelium* leads to a severe phenotype that compromises many aspects of this organism's life cycle as described in detail in our recent work.[15] A summary of the defects and phenotypes are displayed in Table 1. The most dramatic consequence is the inability of the mutant cells to cope with hypoosmotic stress. The membrane lysis observed in the mutant cells under hypoosmotic conditions occurs very rapidly. However, cell swelling is not as noticeable as expected (Fig. 2), suggesting an additional defect in membrane stability. *Dictyostelium* is a soil microorganism that has developed a tubulo-vesicular network of membranes known as the contractile vacuole (CV) that fills up and expels water to the exterior of the cell. Our analysis by transmission electron microscopy and specific CV markers shows a defect in the biogenesis of this organelle.

It is well known that organelle biogenesis, including that of the CV system, is dependent on the secretory pathway. Membrane and protein components traffic from the endoplasmic reticulum and Golgi apparatus to their destinations in the different organelles and the plasma membrane.[17-19] Since Vmp1 is an ER-resident protein, it might function at the initial steps of the secretory pathway and consequently might have pleiotropic effects in other organelles. The rest of the phenotypes observed by the lack of Vmp1 might perfectly fit in the context of a secretory pathway defect including the apparent instability of the plasma membrane as described previously in other reports.[20,21]

The autophagy defect in the *Dictyostelium* vmp1 mutant can also be seen in this context. We observe the accumulation of aberrant double-membrane structures in the mutant that is consistent with abnormal autophagic vesicles. The origin and the assembly of the autophagosome is not completely understood and it is conceivable that the ER, as suggested by our results, might be required for the correct assembly or maturation of the phagosome, perhaps by supplying membrane components. Interestingly, a more direct effect in autophagosome formation has been described in mammalian cell culture. In this case Vmp1 is localized in autophagosomes where it interacts with Beclin 1, an essential autophagy protein.[5] Whether or not *Dictyostelium* Vmp1 behaves in the same way remains to be determined.

Concluding Remarks and Future Perspective

The study of the basic function of new proteins with no characterized motifs is difficult and we must take advantage of any available experimental tool. *Dictyostelium* have taught us a few new important things to be considered about Vmp1 and the consequences of its loss-of-function. We have learned that Vmp1 can play an essential role from the ER, having an impact upon many different membrane traffic-dependent processes. However, many questions remain, such as what affects mammalian Vmp1 sub-cellular localization. Is Vmp1 located in the phagosome or plasma membrane depending on the cell type studied and/or the cellular context? Is Vmp1 located in the ER in any cell type or situation in a similar way as seen in lower eukaryotes such as *Dictyostelium*? What is the relationship between Vmp1 localization and function? Our first glimpse into the function of this important conserved protein is intriguing and now the possibility to address its molecular function and its relevance in human disease is within reach.

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CAPÍTULO 3: AUTOPHAGY DYSFUNCTION AND UBIQUITIN-POSITIVE PROTEIN AGGREGATES IN *DICTYOSTELIUM* CELLS LACKING *VMP1*

En este tercer trabajo nos centramos en la relación de Vmp1 con autofagia y en el propio funcionamiento de la autofagia en *Dictyostelium discoideum*.

En primer lugar y dado que el retículo endoplásmico representa una de las hipótesis más claras como posible donador de membranas para la formación del autofagosoma, que Vmp1 es una proteína localizada en el retículo endoplásmico y que además parecía estar relacionada con la formación de autofagosomas, decidimos estudiar una posible colocalización de Vmp1-GFP con el marcador autofágico RFP-Atg8. Observamos una colocalización parcial de Vmp1-GFP con RFP-Atg8 que sugiere una relación funcional entre el retículo endoplásmico y la formación de los autofagosomas.

Para poder examinar en profundidad la relación de Vmp1 y autofagia se analizaron dos procesos dependientes de autofagia como la supervivencia en ayuno y la formación de células tallo *in vitro*. Las células "wild type" sobreviven 5 días en ayuno y forman células tallo mientras que las células mutantes mueren a los 3 días en ayuno y no son capaces de formar células tallo.

El patrón del marcador autofágico GFP-Atg8 en células humanas y levaduras es un criterio fundamental para determinar la funcionalidad de la autofagia. Células "wild type" y mutantes de *vmp1* fueron transformadas con la construcción GFP-Atg8 y visualizadas por microscopía confocal. Durante el crecimiento normal de células "wild type" predomina un patrón punteado con alguna estructura autofágica vesicular, sin embargo en solución de ayuno predominan las estructuras autofágicas mientras que el número de puntos disminuye ostensiblemente. Sin embargo en células mutantes de *vmp1* el patrón tanto en crecimiento como en ayuno es similar, produciéndose una clara acumulación y agregación de todo el marcaje de GFP-Atg8.

Imágenes de inmunocitoquímica en microscopía confocal mostraron posteriormente que estos agregados de GFP-Atg8 colocalizan con proteínas ubiquitinadas.
Comprobamos por "western-blot" que estas proteínas ubiquitinadas tenían un perfil muy insoluble y caracterizamos la composición de éstas por MALDI-TOF encontrando entre otras el homólogo de la proteína humana p62 en *Dictyostelium discoideum*.

La presencia de agregados proteicos ubiquitinados en la cepa mutante de *vmp1* y la disponibilidad de otros mutantes autofágicos nos dio la posibilidad de testar si éstos también tenían agregados proteicos ubiquitinados. El resultado fue muy interesante ya que parece existir una correlación positiva entre el tamaño de los agregados de los mutantes autofágicos y los defectos fenotípicos que muestran. La cepa mutante en *atg1* tiene un perfil similar a *vmp1* en tamaño de agregados y severidad de defectos fenotípicos, *atg5* y *atg7* muestran agregados más pequeños y defectos fenotípicos menos severos y *atg6* y *atg8* no presentan agregados detectables y sus defectos fenotípicos son aún menos severos.

BASIC RESEARCH PAPER

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Autophagy dysfunction and ubiquitin-positive protein aggregates in Dictyostelium cells lacking Vmp1

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Key words: vacuole membrane protein 1, ubiquitin-positive protein aggregates, autophagy, dictyostelium, Vmp1

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescence protein; RFP, red fluorescence protein; PDI, protein disulfide isomerase; VMP1, vacuole membrane protein 1; cAMP, cyclic adenosine monophosphate; DIF-1, differentiation inducing factor-1; ACD, autophagic cell death

Ubiquitin-positive protein aggregates are a hallmark of many degenerative diseases. Their presence can be induced by dysfunction in protein degradation pathways such as proteasome and autophagy. We now report several lines of evidence suggesting a defect in autophagy in Dictyostelium cells lacking Vmp1 (vacuole membrane protein 1), an endoplasmic reticulum (ER)-resident protein involved in pathological processes such as cancer and pancreatitis. *vmp1*$^-$ null cells are unable to survive starvation or undergo autophagic cell death under the appropriate inductive signals. Moreover, confocal studies using the autophagy marker Atg8 and previous transmission electron microscopy analysis showed defects in autophagosome formation. Although Vmp1 is localized in the ER, we found colocalization with Atg8 suggesting a contribution of both Vmp1 and ER in autophagosome biogenesis or maturation. Interestingly, *vmp1*$^-$ *mutant* cells showed accumulation of huge ubiquitin-positive protein aggregates containing the autophagy marker GFP-Atg8 and the putative Dictyostelium p62 homologue as described in many degenerative human diseases. The analysis of other Dictyostelium autophagic mutants (*atg1*$^-$, *atg5*$^-$, *atg6*$^-$, *atg7*$^-$ and *atg8*$^-$) showed a correlation in the severity of their corresponding phenotypes and the presence of ubiquitin-positive protein aggregates suggesting that the deleterious effects associated with development of these aggregates might contribute to the complex phenotypes observed in autophagy deficient mutants. Our results suggest that Vmp1 is required for the clearance of these ubiquitinated protein aggregates through autophagy and highlight a potential role for Vmp1 in protein-aggregation diseases.

Introduction

Many neurodegenerative diseases are associated with the presence of intracellular or extracellular protein aggregates. These disorders include Parkinson disease (PD), Alzheimer disease (AD), polyglutamine expansion diseases (PQD) and prion-related diseases among others. Collectively, these pathologies can be considered proteinopathies or protein conformation disorders, but the precise role of the protein aggregates in the pathological outcome is a matter of intense debate.[1] It is therefore important to identify key factors involved in the formation, degradation and toxicity of these aggregates at the cellular level. These protein aggregates are composed of primary constituents such as the Aβ (β-amyloid), the protein Tau or α-synuclein. However, the analysis of the protein composition of these brain inclusions have shown a complex and varied composition that might contribute to the pathology.[2] Ubiquitin is frequently present in protein aggregates indicating the activation of protein degradation pathways aimed to prevent the accumulation of misfolded and aggregated proteins. The ubiquitin-proteasome system is devoted mainly to degradation of soluble and short-lived proteins.[3] As insoluble aggregates are poor substrates for the proteasome, autophagy, a lysosomal degradation pathway, is believed to play a major role in their clearance.

Macroautophagy (autophagy hereafter) is a degradative process of cellular components that has been conserved in eukaryotic evolution. In certain circumstances, like starvation or cellular stress, parts of the cytoplasm are included in double membrane vesicles called autophagosomes that fuse later to lysosomes where they are degraded. At the molecular level, several proteins involved in the initial formation and maturation of the autophagosomes have been characterized. These proteins have been identified initially in yeast (named Atg for "Autophagosome proteins"). They are grouped in functional complexes that are necessary for the origin, elongation and completion of the autophagosomes, although the precise mechanism of action of many of these proteins and the way in which they are regulated temporarily are not yet completely understood (recent reviews of the topic include[4-7]). There are at least three signaling complexes required for autophagy: Tor 1 kinase, Atg1 kinase complex and the complex of class III PI3Kinase. Tor 1 controls the nutritional state of the cell regulating protein synthesis and it is also involved in autophagy through the regulation of Atg1. This signaling level is required for the

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induction of autophagy. Tor 1 is in turn being regulated, among other pathways, by the key kinase AMPK, a master regulator of the cellular energetic metabolism.[8,9] The PI3K complex is composed of a ser/thr kinase (Vps34), Vps15 and a coiled-coil containing protein named Vps30/Atg6 (Beclin 1). The activation of this complex is necessary for the recruitment of additional proteins to the autophagosome membrane allowing its elongation.

Autophagy is also induced in other circumstances like the elimination of protein aggregations, organelles or bacteria and it is therefore of immense importance in diverse pathological processes as well as in aging.[7,10,11] Loss of autophagy caused the intracellular accumulation of polyubiquitinated protein aggregates.[12,13] Formation of these aggregates and their clearance by autophagy are dependent on p62/SQSTM1, an ubiquitin-binding scaffold protein that links polyubiquitinated proteins to the autophagic machinery trough the direct interaction with atg8 and polyubiquitin.[14-17]

The social amoeba *Dictyostelium discoideum* is increasingly being used as a simple model to address biological problems that are relevant to human health.[18] Its genetic tractability and the presence of many conserved genes that are absent in *Saccharomyces cerevisiae* make Dictyostelium a valuable simple model for studying the function of new proteins.[19,20] Regarding the study of protein aggregates, Dictyostelium has been used to address the formation of Hirano bodies, actin-rich inclusions frequently associated with neurodegenerative diseases.[21-23] Interestingly, Hirano bodies can be cleared by autophagy in Dictyostelium.[24]

Dictyostelium cells feed on bacteria by phagocytosis and remain in the form of individual cells while bacteria are present. However, when bacteria are exhausted, starvation triggers a process of cellular aggregation and development leading to the formation of fruiting bodies containing spores that allow Dictyostelium to survive.[25] Autophagy is essential for Dictyostelium to get through starvation and complete its developmental program.[26] Multiple origins of nascent autophagosomes are formed in mammals and Dictyostelium, in contrast with *S. cerevisiae* where they are concentrated in a single location of the cytoplasm (called PAS, "Pre-autophagosomal structure or phagosome assembly site"). Autophagosomes appear as a punctate pattern in the cytoplasm when they are analyzed using specific autophagosome markers like Atg8.[27,28] Autophagy mechanisms are highly conserved and the genes involved are present in Dictyostelium and humans. The function of many of these genes has been studied in Dictyostelium.[26-30] Since autophagy in this model system is necessary for several aspects of its life cycle (such as survival under starvation and the completion of its developmental program), the lack of this process leads to phenotypes that are easily recognized. Intriguingly, Dictyostelium autophagy mutants vary in the severity of their phenotypes and the precise cause of these differences are not known.

Fruiting body formation in Dictyostelium requires the differentiation of cells into stalk and spores. The spores will maintain the life cycle by germinating when environmental conditions are adequate. In contrast, stalk cells sacrifice themselves to allow a better dispersal of their siblings. Stalk cells die by a specific programmed cell death that has the characteristics of autophagic cell death.[31-34] In vitro induction of this cell death in Dictyostelium requires at least three components including starvation and the presence of the extracellular signaling molecules cAMP and DIF-1.[34-37]

Vmp1 is a Dictyostelium protein identified in a functional genomic study[19,20,38] and highly similar proteins can be recognized in other organisms including human but it is absent in fungi.[39] The social amoeba is consequently the simplest experimental model to study this protein. Moreover, the expression of a Vmp1 mammalian homologue in the Dictyostelium mutant is able to complement the phenotype suggesting a functional conservation across species and validates Dictyostelium as experimental model to analyze Vmp1 function.[38] Besides the presence of several putative transmembrane domains, Vmp1 does not show any recognizable functional motif and this has complicated its study. As a matter of fact, the function of mammalian Vmp1 is controversial since it has been involved in unrelated processes such as autophagy and cell adhesion.[40,41] In Dictyostelium Vmp1 is localized in the endoplasmic reticulum and has been involved in diverse membrane-traffic-dependent processes.[38,39]

In this study we show that Vmp1 is required for autophagy in Dictyostelium and its absence causes defects in autophagic cell death and the accumulation of ubiquitin-positive protein aggregates. These aggregates contain, among other proteins, p62 and Atg8, revealing a remarkable similarity to other ubiquitin-positive aggregates found in neurodegenerative diseases. We hope that this simple cellular model will shed some light on the basic principles governing autophagy, protein aggregation and cell survival. We have extended our studies to other well-characterized autophagy Dictyostelium mutants to show that protein aggregates are formed in those mutants with a stronger developmental phenotype. Remarkably, *vmp1*$^{-}$ mutant cells show the most severe phenotype among the autophagy mutants, stressing its potential role in human disease.

Results

Vmp1 localization and its role in starvation and autophagic cell death. Our previous studies in Dictyostelium *vmp1*$^{-}$ mutant suggested its possible role in autophagy. In order to further validate this hypothesis, we first wanted to determine if Vmp1 is necessary for autophagy-dependent processes that are essential for Dictyostelium such as cell survival under starvation and the induction of autophagic cell death (ACD). ACD takes place during the terminal differentiation of stalk cells in Dictyostelium development and is necessary for the correct formation of the stalk, a structure required for fruiting body formation and spore dispersal. ACD can be induced in vitro under the appropriate extracellular signals.[32]

Wild-type (WT) and mutant cells were washed free of nutrients and resuspended in PDF buffer for starvation survival assay. Cell viability at different times was measured by scoring the colony-forming units after plating the cells in association with bacteria. A dramatic reduction in cell viability for the mutant cells was observed within the first three days while the WT strain retained high viability for the same period of time (**Fig. 1A**).

When starvation is combined with cAMP and DIF-1 stalk cells differentiate in vitro and die by a process dependent on autophagy.[36] As shown in **Figure 1B**, control wild-type cells under starvation formed small aggregates of cells surrounded by extracellular sheath (marked by an asterisk). However, when wild-type cells were starved and treated with cAMP and DIF-1 they differentiated and became highly vacuolized, a specific feature of ACD and a previous step before membrane disruption. Conversely, mutant cells were unable to aggregate and remained as single cells under control conditions. They were also insensitive to the induction of ACD by the treatment with cAMP and DIF-1 as evident by the absence of vacuolization. In accordance with these results Dictyostelium development is completely blocked at the aggregative stage either in filter conditions or in association with bacteria.[38]

It has been previously described that Vmp1 colocalized with Atg8, a marker of autophagosomes and induced autophagosome formation in pancreatic acinar cells.[42] Therefore, we next asked whether or not Vmp1 is localized in autophagosomes in Dictyostelium cells. Our own previous studies using a strain expressing Vmp1 fused to GFP showed colocalization with the ER marker PDI.[38] The same strain expressing Vmp1-GFP was now transformed with the autophagosome marker RFP-Atg8. The resulting strain showed a characteristic autophagosome punctate pattern as described previously.[26] Cells from this strain were prepared for immunofluorescence to detect PDI. Confocal analysis showed colocalization of Vmp1 with the ER-marker PDI as described previously (**Fig. 2**). Interestingly, a proportion of the RFP-Atg8-positive structures showed a clear colocalization with Vmp1-GFP and PDI as shown in **Figure 2**. After counting the signals of at least 50 cells we found 50% colocalization between Vmp1 and the markers both during growth (HL5) and starvation conditions (PDF). These observations indicate that Vmp1 in Dictyostelium is present in the ER and autophagosomes suggesting a possible role in autophagosome biogenesis or maturation.

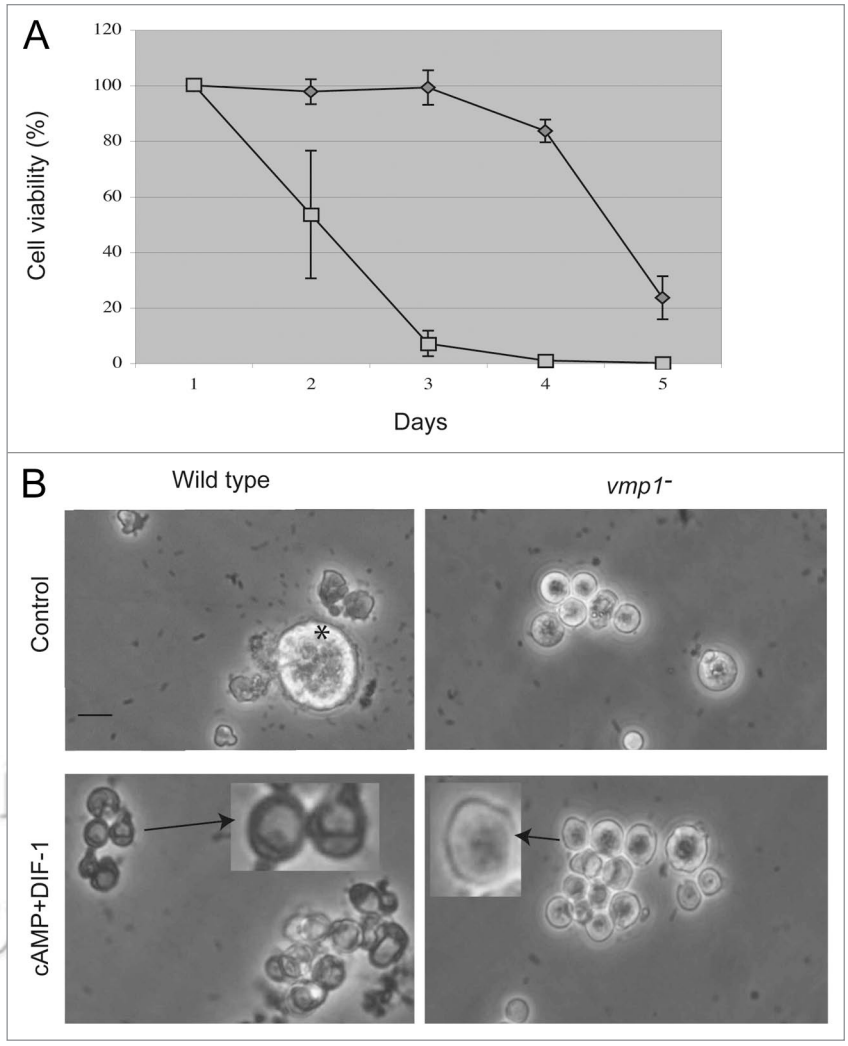

Figure 1. Vmp1 is required both for survival upon starvation and autophagic cell death in Dictyostelium. (A) Dictyostelium WT and mutant cells were washed free of nutrients and maintained in starvation for 5 days. At the indicated times viability of the cells were measured. Viability of the mutant cells dropped very rapidly. The data correspond to three independent experiments. Bars represent the standard deviation of the mean. (B) Autophagic cell death was induced in WT and *vmp1- mutant* cells. Control conditions correspond to cells kept in starvation for the same period of time without treatment. Under these control conditions (starvation in buffer), WT cells remained as single cells except for some aggregation centers (marked by an asterisk) composed of cells embedded in extracellular sheath. Mutant cells did not show any sign of aggregative behavior remaining as single round cells. WT cells treated with cAMP and DIF-1 formed a huge vacuole as they differentiated to stalk cells. The mutant cells did not show vacuolization. Bar: 10 μM.

Aberrant autophagosome formation in cells lacking Vmp1. The localization of Vmp1 in autophagosomes and its role in cell survival under starvation, autophagic cell death and development suggested a possible role in autophagosome formation. To test this hypothesis *vmp1* gene was disrupted in a strain expressing the autophagic marker GFP-Atg8. The resulting strain showed the expected phenotype as previously described (data not shown). Wild-type and mutant strains expressing GFP-Atg8 were analyzed in vivo by confocal microscopy. It should be noted that in vivo analysis provided a better resolution of autophagosome visualization than that obtained in fixed cells. This allowed us to detect slight differences in the fluorescent pattern in wild-type cells between growth conditions (HL5) and starvation (PDF). During growth there was a very dynamic pattern, mostly formed by puncta (**Fig. 3A**). However, during starvation the autophagosomes became larger and most of them showed a clear vesicle appearance (**Fig. 3B**). A count of the fluorescence signal in 35 cells taken randomly in each condition showed that while only 29% of the signal were vesicle-like in HL-5, this proportion increased to 61% in starvation (PDF) conditions. However, the fluorescence pattern in mutant cells was clearly different showing

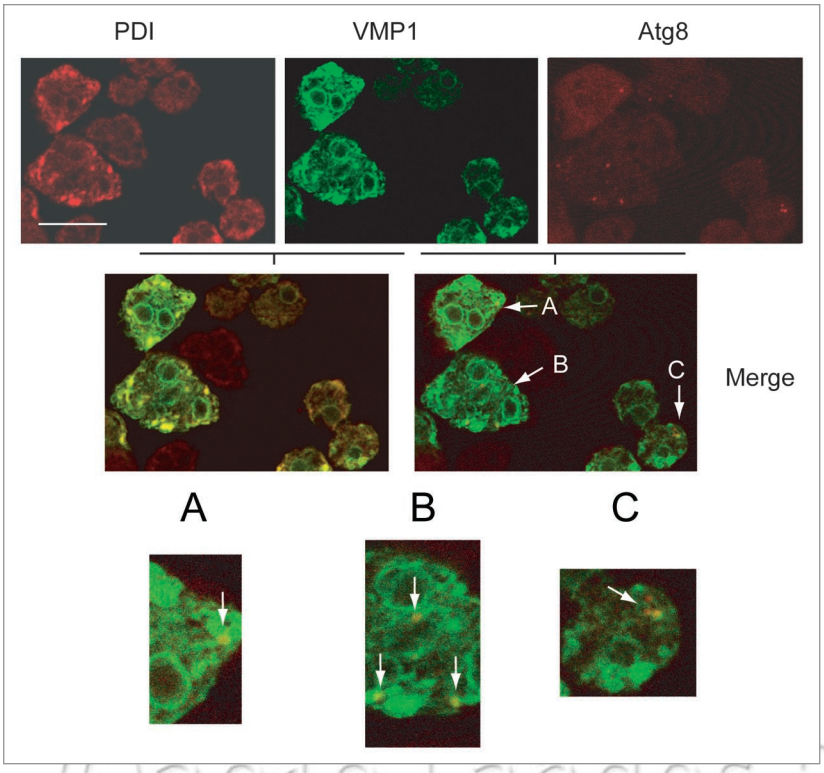

Figure 2. Vmp1 colocalizes with autophagosome marker RFP-Atg8. Cells expressing Vmp1-GFP and RFP-Atg8 were prepared for immunolocalization of PDI, an ER specific marker. A secondary antibody conjugated with far-red Alexa-647 was used to discern the three fluorophores simultaneously by confocal microscopy. To facilitate the visualization of the experiment the fluorescence corresponding to PDI and Atg8 (both in red) was merged to that of Vmp1 (green) in a sequential two-step fashion. High magnification of the cells from the merged Vmp1-Atg8 (A–C) are displayed below to show the colocalization. Bar: 10 μM.

a huge accumulation of GFP-Atg8, irrespective of the growth or starvation conditions. Video-lapse of mutant cells clearly showed the static nature of the GFP-Atg8 signal (**Suppl. movie 1**) with no formation of vesicle-like autophagosomes. In contrast, the WT cells in a similar temporal framework showed a very dynamic pattern of multiple foci moving rapidly and forming vesicle-like structures (**Suppl. movie 2**).

Ubiquitin-positive protein aggregates in *vmp1*⁻ mutant cells. There is a growing body of evidence that autophagy dysfunction might contribute to the accumulation of protein aggregates in aging and disease. These aggregates are often associated with ubiquitin suggesting that degradative pathways are being activated to avoid the deleterious effect of their accumulation. We have now addressed this issue in Dictyostelium *vmp1*⁻ mutant and other autophagy-deficient strains. Our aim was to determine the interplay between autophagy and protein accumulation and gain insight into a potential role of Vmp1 in proteinopathies.

Interestingly, as shown in **Figure 4A**, Dictyostelium *vmp1*⁻ mutant cells showed huge ubiquitin-positive aggregates as determined by immunofluorescence confocal microscopy using a specific antibody (**Fig. 4A**). The large size of the aggregate might facilitate their fractionation into an insoluble detergent-resistant fraction. To address this, cells were lysed and incubated with a buffer containing 0.1% NP40 and 1% TritonX-100 as described in materials and methods. After a low-speed centrifugation the supernatant and the pellet were subjected to western blot analysis to detect ubiquitinated proteins. As shown in **Figure 4B**, a typical signal marking a wide region of high molecular size proteins was accumulated in the pellet fraction of the mutant cells. This simple method confirmed the insolubility of these structures and allowed us to identify some of the proteins present in the aggregates (as explained below).

We next wanted to determine if these ubiquitinated protein aggregates colocalized with the aberrant accumulation of the autophagosome marker GFP-Atg8 described in the mutant cells. WT and *vmp1*⁻ mutant strains expressing GFP-Atg8 were used to detect ubiquitinated proteins by immunofluorescence (**Fig. 5A**). WT staining using the anti-ubiquitin antibody gave a fairly uniform signal that did not colocalize with autophagosomes. However, the mutant strain showed a clear colocalization of these markers suggesting that GFP-Atg8 is being recruited to these ubiquitin-positive aggregates. We next asked whether or not these aggregates colocalize with lysosomes as this would indicate a functional autophagy flux. As expected colocalization was not found between the aggregates and lysosomes as detected by lysotracker staining (**Fig. 5B**).

Autophagy is required for the clearance of ubiquitin-positive protein aggregates. Dictyostelium is a suitable model for the study of autophagy and several mutants affecting key autophagy genes have been generated.[26,27] The multicellular development of this organism is affected by autophagy dysfunction but the severity of the phenotype was found to vary among the mutants. It is not clear how differences in the level of autophagy can affect specific aspects of development. The presence of protein aggregates is believed to alter cell function in many different ways. The cause of these effects is not well understood but several lines of evidence suggest that aggregates might sequester proteins required in other cellular functions, in addition to the possible steric effects that might affect cell movement or intracellular traffic. We wanted to determine whether ubiquitin-positive aggregates are also present in other well-defined autophagy mutants and whether or not the severity of their phenotype correlate with the presence of protein aggregates.

We have used Dictyostelium *atg1*⁻ mutant cells and *atg1*⁻ mutant cells expressing GFP-Atg8 to study the presence of ubiquitinated-protein aggregates by immunofluoresce and western blot. As previously described for *vmp1*⁻ mutant, we found the presence of large ubiquitin-positive aggregates in *atg1*⁻ cells and colocalization of these aggregates with GFP-Atg8 (**Fig. 6A**). The size of the aggregates and the proportion of cells containing them were not as prominent as in *vmp1*⁻ cells. The presence of these ubiquitin-protein aggregates was also tested by immunofluorescence in Dictyostelium *atg5*⁻, *atg6*⁻, *atg7*⁻ and *atg8*⁻ mutants. As shown in

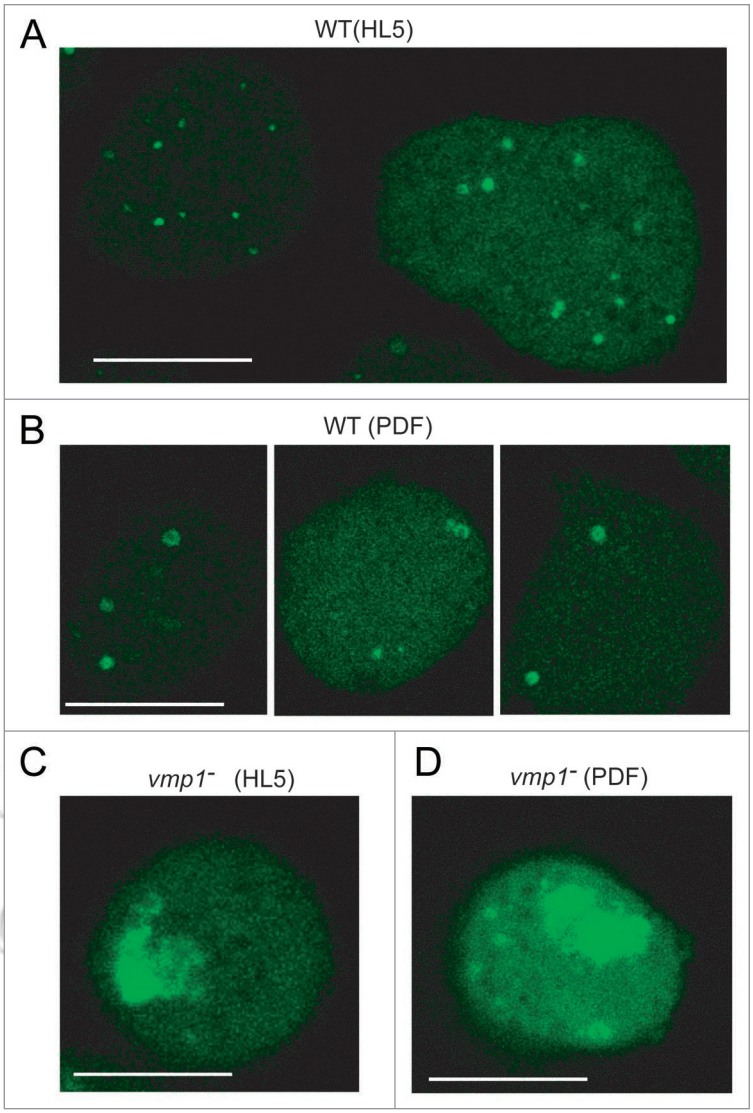

Figure 3. Aberrant autophagosome formation in *vmp1*- Dictyostelium cells. In vivo confocal microscopy of GFP-Atg8 autophagosome marker in WT and *vmp1*- cells. (A and B) Wild-type cells show a different pattern under growth (HL5) or starvation (PDF) conditions. During starvation cells had fewer structures but they were larger and most of them showed cup-like or vesicle-like appearance. However, during growth conditions most of the signals displayed a punctate pattern. (C and D) Mutant cells showed a strong aberrant GFP-Atg8 fluorescence under growth or starvation conditions. Bar: 10 μM.

Figure 6B, *atg5*- and *atg7*- mutants had ubiquitin-positive accumulations. However these aggregates were not as noticeable as in *vmp1*- or *atg1*- mutants. Ubiquitin-positive aggregates were not detected in *atg6*- and *atg8*- mutant cells (the later not shown). Detection by western blot of ubiquitinated proteins in the pellet fraction after detergent cell lysis only showed clear accumulation in *atg1*- mutant. Interestingly, there is a good correlation between the severity of the phenotype of the different mutants and the presence of these protein aggregates as shown in **Table 2**.

Protein aggregates contain ubiquitin and other ubiquitin-related proteins. As we have shown above, the ubiquitinated protein aggregates present in *vmp1*- *mutant* cells can be separated by a low-speed centrifugation after detergent lysis. We took advantage of this property to identify proteins present in the aggregates. Wild-type and mutant cells were lysed under those conditions, centrifuged and the pellet solubilized in SDS-loading buffer. High molecular proteins were separated by SDS-PAGE and the gel stained with commassie. We found a strong enrichment in protein bands in the mutant pellet that were barely visible in wild type (data not shown). Bands were excised and processed for MALDI-TOF analysis. **Table 1** shows a list of the identified proteins. As expected, we found the presence of ubiquitin, presumably bound to other proteins of high molecular size. Interestingly, the analysis detected the presence of a putative Dictyostelium homologue of p62 (DDB_G0292188), a protein involved in directing ubiquitinated protein aggregates to autophagosomes and, like ubiquitin, is often found accumulated in protein aggregation diseases. A comparative analysis of DDB_G0292188 primary sequence with the human p62 is shown in **Supplementary**

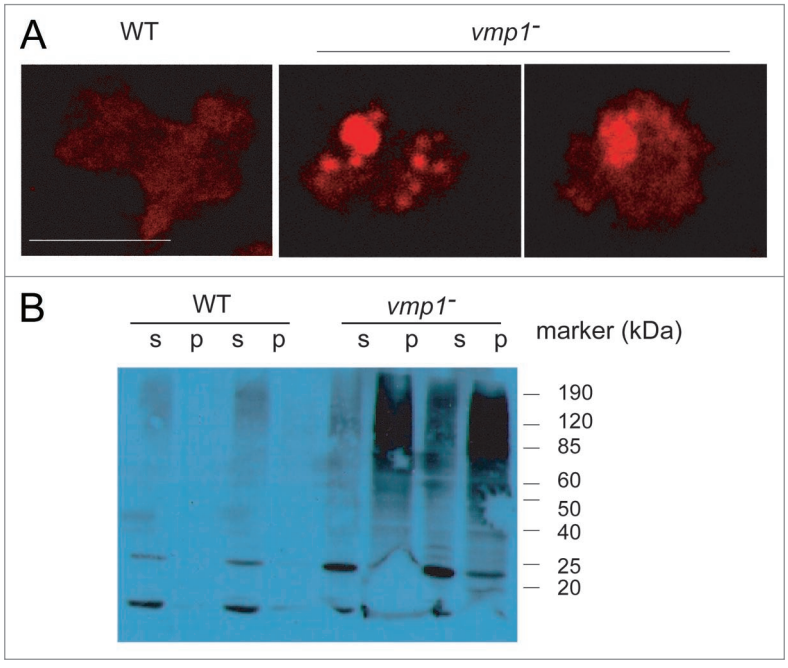

Figure 4. Ubiquitin-positive protein aggregates in *vmp1*- mutant. (A) Ubiquitin-positive aggregates were analyzed by immunofluorescence using anti-ubiquitin antibody. WT showed a uniform staining while most of the mutant cells showed huge accumulations. (B) WT and mutant cells were lysed in buffer containing 1% Triton X-100 and 0.1% NP-40. After two centrifugation steps the pellets and supernatants were separated by SDS-PAGE and transferred to a filter for western blot to detect ubiquitinated proteins. A representative experiment of two independent samples is shown. The lanes corresponding to the protein pellets of the mutant strain showed a strong signal at high molecular size. Bar: 10 μM.

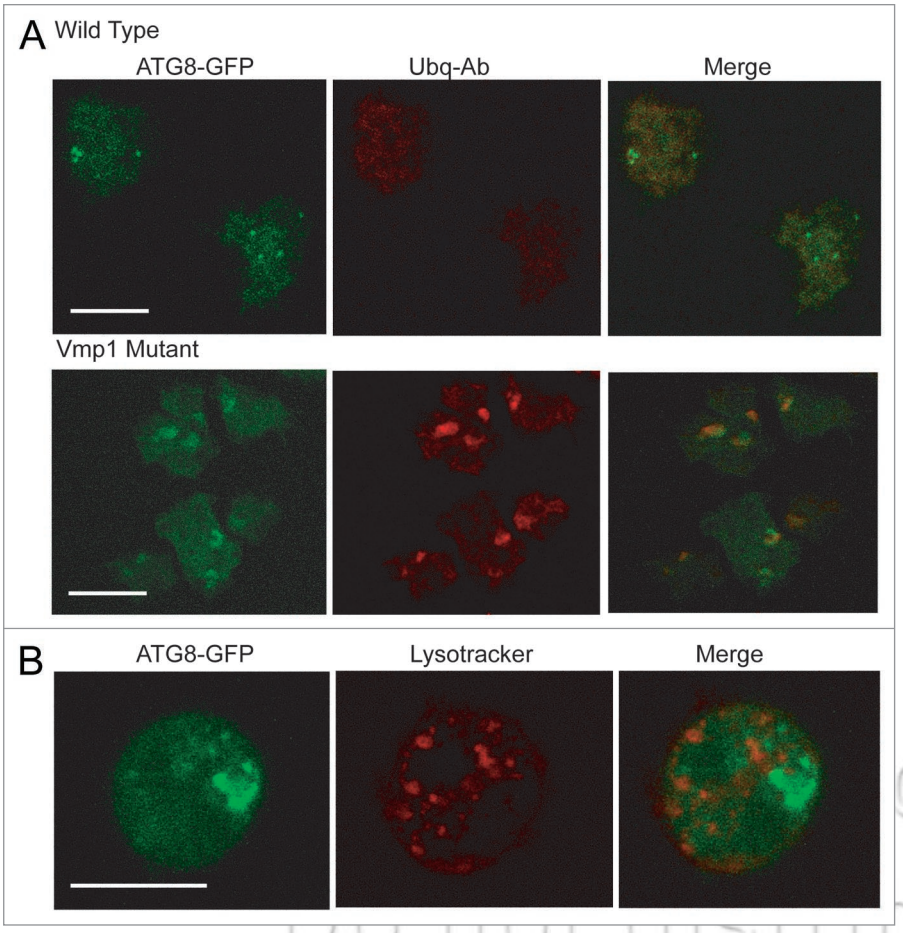

Figure 5. GFP-Atg8 marker colocalizes with ubiquitin-positive aggregates. (A) WT and *vmp1*⁻ mutant strains expressing the autophagosome marker GFP-Atg8 were prepared for immunodetection of ubiquitin-positive protein aggregates. Colocalization of both markers can be observed in the mutant aggregates. (B) Lysosomes were labeled with lysotracker in the *vmp1*⁻ mutant strain expressing the autophagosome marker GFP-Atg8. The protein aggregates were not found in close proximity to the lysosomes. Bar: 10 μM.

Figure 1. The Dictyostelium protein contains the characteristic functional motifs present in the human p62 protein such as the PB1 domain required for oligomerization, the ubiquitin-associated domain (UBA) necessary for the interaction with ubiquitin and a ZZ-type zinc finger domain.[43] Other ubiquitin-related proteins have been detected such as DDB_G60269462, an ubiquitin-domain containing protein and DDB_G0284757, an ovarian tumor (OTU)-domain containing protein. The OTU-domain family comprises a group of putative proteases involved in de-ubiquitinated processe.[44,45] Our analysis also detected the presence of other proteins potentially involved in other cellular functions such as DDB_G0291127, a major vault protein; DDB_G0289467, an AAA ATPase domain-containing protein and other proteins of unknown function.

Discussion

Vmp1 is a multispanning membrane protein of unknown molecular function. Its subcellular localization suggests multiple cellular roles not yet fully elucidated. Our results indicate that Vmp1 is an ER-resident protein in Dictyostelium as also described in Drosophila and plants. In Drosophila, Vmp1 (known as TANGO-5) was identified in a functional genomic RNAi screening as a protein required for protein secretion and Golgi organization.[46] Our previous results in Dictyostelium also indicated that Vmp1 is necessary for protein secretion and other membrane-traffic processes.[38] We postulate a possible traffic defect originating from the ER and affecting multiple membrane-traffic-dependent processe.[38] Intriguingly a recent report showed that Vmp1 was located in the plasma membrane playing a role in cell-cell contact in kidney cancer cell line Caki-2.[47] However, localization studies in other mammalian cell lines (HELA and NIH3T3) showed that Vmp1 was located in autophagosomes.[40]

Our results now seem to reconcile in part some of these data. At least in Dictyostelium Vmp1 can be located simultaneously in the ER and autophagosomes suggesting a functional interplay between the ER and autophagy. However, we have not found the protein in the plasma membrane. According to our results in Dictyostelium, a recent report showed evidence that cup-shaped protrusions from the ER, the omegasomes, might serve as platforms for autophagosome formation in mammalian cells.[48,49] Moreover, it has been described that ER-phagy, a form of autophagy that selectively include ER membranes, might use ER as a membrane source for autophagosome biogenesis.[50] The interplay between the ER and autophagy has also been revealed in yeast where membrane traffic from the ER is somehow required in autophagosome formation.[51] The physical presence of Vmp1 in a proportion of autophagosomes leave open the possibility of a direct role, perhaps by supplying or regulating membrane constituents. In agreement with this hypothesis a protein-protein interaction of Vmp1 with Beclin 1, a mammalian autophagy initiator, has been reported in mammalian cells.[40] More work will be required to test these hypotheses and determine the precise molecular function of Vmp1 in autophagy.

Autophagy is required for several aspects of Dictyostelium life cycle including survival of starvation, morphogenesis and differentiation into stalk cells, a process that takes place by autophagic cell death.[26,27,34,35,52] We have now shown that all these aspects are dependent on Vmp1. Confocal analyses with the autophagic marker GFP-Atg8 showed an aberrant pattern suggesting a defect in autophagosome formation. In accordance with this observation, electronic microscopy studies in *vmp1*⁻ cells failed to detect normal autophagosomes.[38]

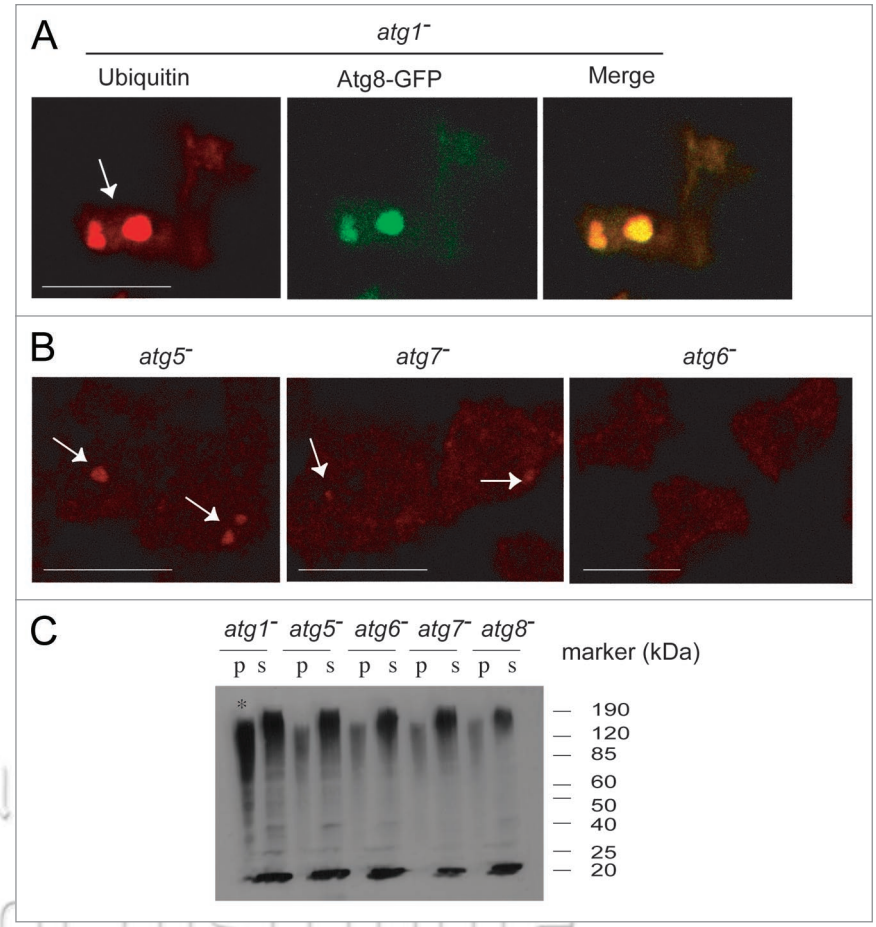

Figure 6. Presence of ubiquitin-positive protein aggregates in Dictyostelium autophagy mutants. (A) Ubiquitin-positive protein aggregates were detected by confocal immunofluorescence in *atg1*- cells expressing the autophagosome marker GFP-Atg8. (B) Presence of ubiquitinated-protein aggregates in *atg5*-, *atg7*- and *atg6*- mutants. While *atg5*- and *atg7*- mutants showed smaller protein aggregates, *atg6*- mutant cells (and *atg8*- mutant, not shown) did not show any. Bar: 10 μM.

Dictyostelium cells, in contrast with the yeast model and similarly to mammalian cells, have multiple autophagosome origins displaying the typical punctate pattern when studied using the marker Atg8. Unexpectedly, this punctate pattern is also present under growth conditions while classical TEM studies have shown that the formation of autophagosome vesicles in Dictyostelium was only triggered by starvation and few autophagosomes were detected during growth.[53] Our in vivo confocal studies in wild type have shown a change in the punctate pattern between growth and starvation that might explain this discrepancy. During growth we found that most of the GFP-Atg8 signals displayed a punctate appearance and only few vesicle-like autophagosomes could be clearly observed. However, during starvation, the number of puncta was reduced and the vesicle-like appearance of the autophagosomes became more evident. The punctate pattern observed under growing conditions might reflect initial stages of autophagosome formation. The completion of these foci into vesicle-like autophagosomes would probably require additional signaling events triggered by starvation. Additional work would be required to test this hypothesis. In any case, this normal pattern observed in wild type was severely altered by the lack of Vmp1 as the autophagosome marker appeared aggregated both under growth and starvation conditions.

Protein aggregation has an enormous interest as it is involved in many human diseases. The presence of mutated aggregate-prone proteins or dysfunction in protein degradation pathways, including autophagy, can contribute to this phenotype. We now report that the lack of Vmp1 in Dictyostelium leads to the accumulation of detergent-resistant protein aggregates containing ubiquitinated proteins. We have partially characterized these aggregates to determine the presence of other proteins. Interestingly, among the identified proteins we found DDB_G0291127, a putative Dictyostelium homologue of p62. This hypothesis is supported by sequence comparison since p62 in Dictyostelium has not yet been characterized functionally. DDB_G0291127 is the most similar protein to mammalian p62 at the amino acid level and more importantly, it contains the expected functional motifs required for p62 multimerization and interaction with Atg8 and ubiquitin. Interestingly GFP-Atg8 was also found to be present in the protein aggregates by confocal analysis. In mammalian cells p62 plays a key role in the formation of ubiquitinated aggregates and provides a link with the autophagic machinery by its interaction with Atg8. Our data suggest the possibility of a similar mechanism in Dictyostelium. The inability of *vmp1*- cells to clear these aggregates by autophagy would explain their accumulation as described in mouse models where autophagy has been disturbed by mutations in *atg5* and *atg7*.[13,54]

It should be noted that Atg8, that we have observed to be present in the aggregates by means of confocal microscopy was not detected by MALDI-TOF of partially purified aggregates. It is

Table 1. Proteins identified by MALDI-TOF in the protein aggregates

Dicty-base entry	Domains
DDB_G0289467	AAA ATPase domain-containing protein
DDB_G0276361	Hypothetical protein of unknown function
DDB_G0291127	Major vault protein
DDB_G0282295	Ubiquitin
DDB_G0288947	Hypothetical protein of unknown function
DDB_G0284757	OTU domain-containing protein
DDB_G0269462	Ubiquitin domain-containing protein
DDB_G0291127	Ubiquitin-associated (UBA) domain-containing protein
DDB_G0292188	Hypothetical protein of unknown function
DDB_G0269482	Hypothetical protein of unknown function

Table 2. Autophagy mutants in dictyostelium and their phenotype

Mutant	Ubiq. + aggregates	Growth	Starvation survival	Development	References
vmp1$^-$	+++	+++	+++	+++	Calvo-Garrido et al. 2008
atg1$^-$	+++	++	+++	+++	Otto et al. 2004
atg5$^-$	++	-	++	++	Otto et al. 2003
atg7$^-$	+	-	++	++	Otto et al. 2003
atg6$^-$	-	-	++	+	Otto et al. 2004
atg8$^-$	-	-	++	+	Otto et al. 2004

+++, severely affected; ++, affected; +, slightly affected; -, similar to wild type.

possible that our simple experimental conditions might solubilize some of their constituents. Further analysis using different conditions and more sophisticated protein purification protocols would be necessary for a comprehensive analysis of the molecular composition of these aggregates.

The similarity of these aggregates with those found in neurodegenerative diseases highlights the interest of Dictyostelium as a cellular model system in proteinopathies and their possible interplay with autophagy. Therefore, we extended our studies to other well-characterized Dictyostelium autophagic mutants. The presence of ubiquitin-positive protein aggregates in *atg1*$^-$ and to a lesser extent in *atg5*$^-$ and *atg7*$^-$ mutants confirmed the importance of autophagy in the clearance of ubiquitinated protein in Dictyostelium. However, their absence in *atg6*$^-$ and *atg8*$^-$ mutants revealed a second layer of complexity as we found a fairly good correlation between the severity of the phenotype and the presence of these protein aggregates. We have summarized these observations in **Table 2** to facilitate the comparison. *vmp1*$^-$ and *atg1*$^-$ mutants show a striking phenotypic similarity as they have reduced growth rates both in axenic media and in association with bacteria, development is blocked in the aggregative stage and the response under starvation is severely compromised. We have now found the presence of huge ubiquitin-positives aggregates in both strains and similar defects in the abnormal morphology of the autophagosome marker GFP-Atg8. However, Dictyostelium *atg5*$^-$ and *atg7*$^-$ mutants show a less severe phenotype. These proteins are part of an ubiquitin-like conjugation reaction where Atg5 is covalently conjugated with Atg12. Both mutant strains showed normal growth in contrast to *vmp1*$^-$ and *atg1*$^-$ mutant strains. Regarding development, although they have defects on aggregation when developed in bacterial lawns, they are able to form multitipped aggregates in filter development. These structures culminate to form small fruiting bodies with no spores. We have found small ubiquitin-positive protein aggregates in both strains.

Atg6, the yeast homologue of mammalian Beclin 1, and Atg8, also known as LC3 (microtubule associated protein light chain 3), show the mildest phenotype among the autophagy mutants. Development is almost normal and although they form spores, their viability is reduced. No ubiquitin-positive aggregates were detected in these strains. Therefore, the complexity of Dictyostelium autophagy phenotypes and the different degree of developmental affectations might be related to the presence of these aggregates.

It has been postulated that the presence of protein aggregates might affect cell function not only by means of its steric effects but also by sequestering other proteins that might be attracted to the aggregate by specific interaction with other existing proteins. Recently the formation of actin inclusions in Dictyostelium cells by mistargeting VASP, an actin-binding protein, to endosomes[23] has been described. These actin aggregates sequester other actin-binding proteins and endosomal proteins promoting their disappearance from the cytosol.[23] In neurodegenerative diseases, besides the major molecular constituents of protein aggregates (usually a mutated aggregate-prone protein), there have been identified other proteins that might contribute to the pathology.[2] The aggregates generated in *vmp1*$^-$ cells contained proteins that might not be related to autophagy or ubiquitin such as DDB_G0291127, a major vault protein, DDB_G0289467, an AAA ATPase domain-containing protein, and other proteins of unknown function. The presence of these proteins in the aggregates might alter their correct localization and concentration in the cell and contribute to the phenotype in intricate ways that remain to be explored.

Materials and Methods

Dictyostelium cell culture, transformation and generation of vmp1 null mutant. Cells were grown axenically in HL5 medium or in association with *Klebsiella aerogenes* in SM plates.[55] The original *vmp1*$^-$ strain was generated in AX4 as described previously.[38] DH1 was the parental strains for the autophagy mutants *atg1*$^-$, *atg5*$^-$, *atg6*$^-$, *atg7*$^-$ and *atg8*$^-$ as well as the GFP-Atg8 expressing strains. They were kindly provided by Dr. Kessin's laboratory.[26,27] Disruption of Vmp1 in DH1 strains showed the same phenotype as previously described in AX4.[38] Transformations were carried out by electroporation as described previously.[56] For starvation assays, cells were resuspended in PDF buffer (20 mM KCl, 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 6.4) for the indicated times and cell viability was determined by counting the number of colony-forming units after plating on SM plates in association with bacteria. Disruption of Vmp1 gene in the Dictyostelium strain expressing GFP-Atg8 was performed as previously described.[38]

Immunocytochemistry and confocal microscopy. For immunocytochemistry, cells were allowed to adhere to coverslips and fixed in 2.5% paraformaldehyde (Polysciences, Inc., 00380) in PBS for 30 minutes. After two washes with PBS, cells were permeabilized with chilled methanol during 2 minutes and incubated during 20 minutes in blocking buffer (0.2% BSA in PBS). The samples were then incubated with the first antibody in

blocking buffer for 1–3 hours. After five washes with blocking buffer the appropriate secondary antibody was added at a dilution of 1/1,000 in blocking buffer for 30 minutes. After three washes with blocking buffer cells were mounted for microscopic observation. Confocal analysis was performed in a Leica TCS SP5 using a PL APO 63X/1.4-0.6 objective and a LAS-AF (Leica Application Suite) software. For excitation of GFP a 488-nm Argon laser was used. PDI antibody (221-64-1 ascitis, mouse monoclonal) was kindly provided by Pierre Cosson from the University of Geneva (Switzerland) and used at a dilution 1:1,000. Ubiquitin monoclonal antibody was from Cell Signalling (3936).

Autophagic cell death assay. Cells were washed free of nutrients in phosphate buffer and deposited in multiwell plates at 5 x 10^5 cells/ml in spore buffer (KCl 20 mM; NaCl 20 mM; $CaCl_2$ 1 mM; $MgCl_2$ 1 mM; MES pH 6.2 10 mM) containing 5 mM cAMP. After 18 hours the media was washed twice with spore medium and incubated with 100 nM DIF-1 (Biomol International, GR324) in spore buffer for 24 hours. Photographs were taken directly in the plates with an inverted Leica microscope.

Detection of ubiquitinated proteins by western blot. 5 x 10^6 cells were resuspended in 100 µl of PBS supplemented with 0.1% NP40 (Sigma, I-3021) and 1% TritonX-100 (Merck, 1.08603.1000) and incubated for 10 minutes on ice. Cells were then passed trough a syringe four times for a complete cell disruption. The extracts were then centrifuge at 4,000 rpm for 5 minutes. Supernatants were kept for further analysis and the pellets resuspended again in the same buffer and subjected to the same process. After the last centrifugation the supernatants were now discarded and the pellets were solubilized directly in 25 µl of SDS loading buffer. Pellets and the first supernatant fractions were separated by SDS-PAGE, transferred to a filter and analyzed by western blot with an anti-ubiquitin monoclonal antibody from cell-signalling (3936).

Enrichment of protein aggregates, in-gel digestion of proteins, MALDI-MS/MS and database searching. Cells were treated as described in the above section and the pellets were separated by SDS-PAGE using a 6% acrylamide concentration to allow the separation of high molecular size proteins. After coomassie blue staining the protein bands were excised manually from the gel and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics). The digestion protocol used was previously described,[57] with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (Plusone, 17-1318-02) in 50 mM ammonium bicarbonate (99.5% purity; Fluka, 09830) and alkylation with 55 mM iodoacetamide (Sigma, I-6125) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Sigma, 34967) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, V5111) at a final concentration of 8 ng/µl in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Fluka, 91699) was added for peptide extraction. An aliquot of the above digestion solution was mixed with an aliquot of cyano-4-hydroxycinnamic acid (Bruker-Daltonics, 205931) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 µm AnchorChip prestructured MALDI probe (Bruker-Daltonics, 209518) and allowed to dry at room temperature. MALDI-MS/MS data were obtained in an automated analysis loop using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT MS/MS device.[58] Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1,000 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Automated analysis of mass data was performed using the flexAnalysis software (Bruker-Daltonics). Internal calibration of MALDI TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3,200 m/z region. MALDI-MS and MS/MS data were combined through the BioTools program (Bruker-Daltonics) to search a nonredundant protein databases (NCBI: National Center for Biotechnology Information; and SwissProt: Swiss Institute for Bioinformatics) using the Mascot software (Matrix Science).[59]

Acknowledgements

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/CalvoGarridoAUTO6-1-Sup.pdf

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CAPÍTULO 4: AUTOPHAGY IN *DICTYOSTELIUM*: GENES AND PATHWAYS, CELL DEATH AND INFECTION

Este cuarto artículo es el último dentro del trabajo en que se centró mi tesis doctoral. Es una revisión que mediante un análisis bioinformático identifica y muestra las proteínas que posiblemente participen en autofagia en *Dictyostelium discoideum*, comparándolas con sus posibles homólogos en humanos y levaduras.

De acuerdo a las proteínas homológas a levaduras y humanos presentes en *Dictyostelium discoideum* y al conocimiento de la bibliografía existente, claramente podríamos dividir la autofagia en *Dictyostelium discoideum* en 4 etapas:

1. Inducción de la autofagia, complejo Atg1.
2. Nucleación y complejo fosfatidilinositol 3-quinasa (PtdIns3K).
3. Expansión vesicular y sistemas de conjugación similares a ubiquitina.
4. Fusión del autofagosoma con el lisosoma.

Además se repasan los primeros trabajos donde *Dictyostelium discoideum* ha servido como organismo modelo en el estudio de la autofagia y su relación con muerte celular, haciendo especial hincapié en la muerte celular por necrosis que aparece en ausencia de la muerte celular por autofagia.

Se describen también estudios anteriores con diferentes patógenos bacterianos como *Salmonella*, *Legionella*, *Pseudomonas* y la utilización de *Dictyostelium discoideum* como huésped y cómo en la infección por *Legionella* la cepa deficientes de *atg9* es mucho más vulnerables que la cepa “wild type”.

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Autophagy in Dictyostelium
Genes and pathways, cell death and infection

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Key words: Dictyostelium, social amoeba, autophagy, autophagic cell death, autophagy and infection, Vmp1, Atg proteins

The use of simple organisms to understand the molecular and cellular function of complex processes is instrumental for the rapid development of biomedical research. A remarkable example has been the discovery in *S. cerevisiae* of a group of proteins involved in the pathways of autophagy. Orthologues of these proteins have been identified in humans and experimental model organisms. Interestingly, some mammalian autophagy proteins do not seem to have homologues in yeast but are present in Dictyostelium, a social amoeba with two distinctive life phases, a unicellular stage in nutrient-rich conditions that differentiates upon starvation into a multicellular stage that depends on autophagy. This review focuses on the identification and annotation of the putative Dictyostelium autophagy genes and on the role of autophagy in development, cell death and infection by bacterial pathogens.

Introducing Dictyostelium, A Suitable Model to Study Autophagy

Dictyostelium discoideum is a simple eukaryote that lives in the soil and feeds on bacteria by phagocytosis. The individual cells divide by binary fission as long as food is present, however, when bacteria are exhausted, starvation triggers a process of chemotaxis driven by cyclic-AMP (cAMP).[1-3] The resulting cell aggregate is surrounded by a complex extracellular matrix of protein, cellulose and polysaccharides that isolates it from the environment. This cellular association behaves like a true multicellular organism undergoing different stages of development accomplished by the coordination of morphogenesis and cellular differentiation. Eventually, the aggregates give rise to fruiting bodies, each formed by a cellular stalk that supports a mass of spores. The latter will germinate when environmental conditions are adequate.[4-6] The life cycle of the experimental model species *Dictyostelium discoideum* and representative pictures of each stage are illustrated in **Figure 1**.

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Since Dictyostelium cells undergo development in the absence of any source of external nutrients they need to mobilize resources to be able to respond to the high cell activity required for aggregation and morphogenesis. This mobilization is in part achieved by glycogenolysis and autophagy, the degradation and turnover of the cells' own biomolecules. Autophagy is essential for development in many different systems.[7-9] Three types of autophagy have been described, chaperone-mediated autophagy, microautophagy and macroautophagy. In the first one, specific proteins are recognized by chaperones that mediate their translocation across the limiting membrane of the lysosome into the lumen for their degradation.[10] This form of selective autophagy plays an important role in the cell's response to stress and the presence of damaged proteins. In contrast, microautophagy consists of the invagination or protrusion/septation of the lysosome membrane, thus capturing the cargo and delivering it into the lysosomal lumen, again for degradation.[11] We will focus our review on the third type, macroautophagy (referred to as autophagy hereafter), a mechanistically different degradative process characterized by the formation of double-membrane vesicles called autophagosomes that engulf part of the cytosol or even organelles. The outer membrane of the autophagosomes subsequently fuses with lysosomes, forming autolysosomes where the contents and inner membrane of the autophagosome are degraded and the simple molecular constituents recycled. This form of autophagy is essential for temporary cell survival under starvation conditions. Autophagy is also induced in other circumstances such as for the elimination of protein aggregates or defective organelles or in response to intracellular bacteria, and it is therefore of immense importance in diverse pathological processes as well as in aging.[12-14] The origin of the autophagosomal membrane and the mechanism mediating its expansion and maturation are not yet completely understood.

In mammals and Dictyostelium, nascent autophagosomes originate in the cytoplasm from multiple origins, in contrast with *S. cerevisiae,* where these structures are concentrated in a single location of the cytoplasm (named the PAS or phagophore assembly site). These autophagosomes appear in Dictyostelium and higher organisms as a punctate pattern in the cytoplasm when they are analyzed by fluorescence microscopy using specific autophagosome markers like GFP-Atg8/LC3.[15,16] At the molecular level, several proteins involved in autophagosome formation

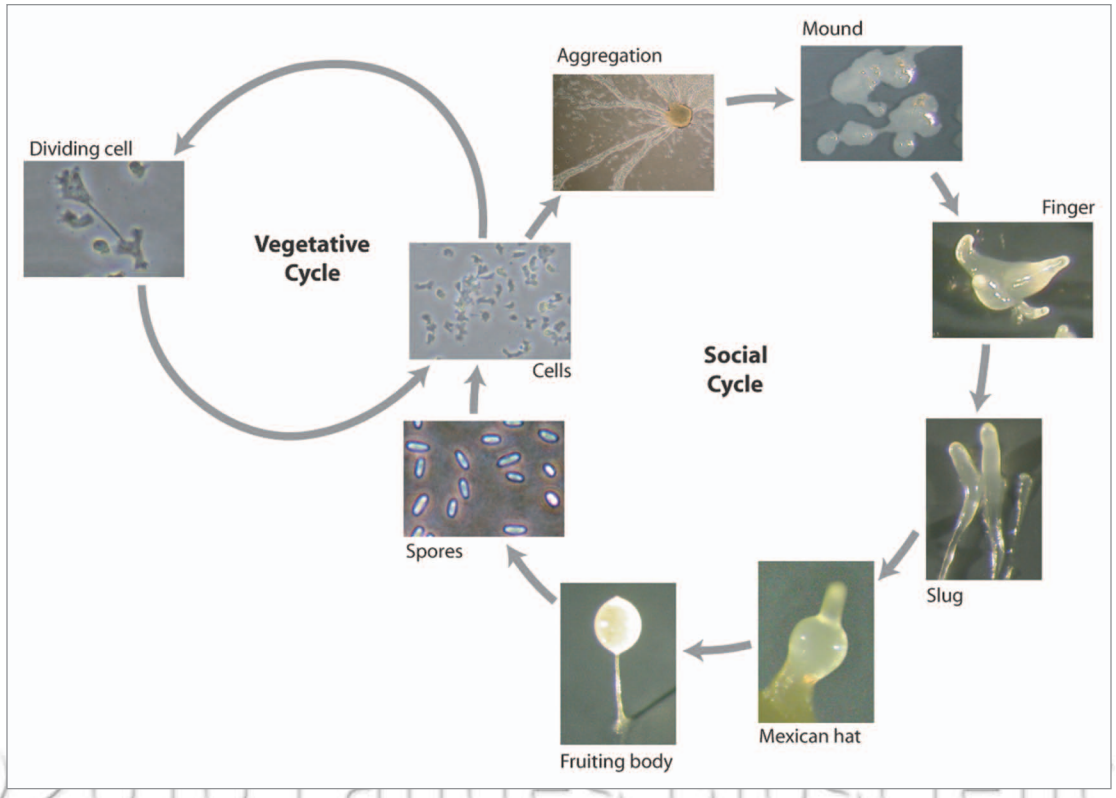

Figure 1. Dictyostelium life cycle. Representative pictures of vegetative and developmental stages are shown. In the wild, Dictyostelium amoeba feed on soil bacteria by phagocytosis but most laboratory strains are also able to grow in liquid axenic media by macropinocytosis. It is important to point out that cells are haploid throughout these vegetative and developmental cycles and this facilitates the generation of knockout strains.

(named Atg for autophagy-related) have historically been identified, primarily in the yeast *S. cerevisiae*. They are grouped in functional complexes required for the origin, elongation, completion and degradation of the autophagosome membrane, although the precise mechanisms of action of many of these proteins and the way in which they are regulated temporally are not yet completely understood (reviewed in ref. 12, 17–19). Two different complexes containing the protein kinase Atg1 and the lipid kinase Vps34 are necessary for induction and nucleation of autophagosomes and to recruit other proteins to the assembly site. Vesicle expansion and completion require two ubiquitin-like conjugation systems involving Atg8 and Atg12. Other proteins like Atg2, Atg9 and Atg18 play a role in membrane traffic and the biogenesis of the autophagosome. Many of these autophagy proteins are conserved in evolution and can be recognized in Dictyostelium by sequence homology analysis as described in detail below.

Despite its simplicity, Dictyostelium shows striking similarities with higher eukaryotes in many biological aspects including chemotaxis,[2,3,20-22] developmental signaling pathways,[4,23,24] the response to bacterial infections,[25-28] the response to therapeutic drugs[29-32] and programmed cell death including autophagic cell death (reviewed in ref. 33). The Dictyostelium genome has been fully sequenced[34] and carefully annotated (http://dictybase.org/) and it is amenable to a wide range of molecular genetic techniques including the generation of mutants by homologous recombination and random genetic screens,[6,33,35-38] that have facilitated the use of comparative genomics to identify relevant genes conserved in the human genome.[37,39]

General Autophagy Mechanisms and Evolutionarily Conserved Autophagy Genes: Induction of Autophagy and the Atg1 Complex

We will now examine the potential of Dictyostelium as a model for autophagy by describing the proteins that are known to be involved in this complex process in other systems and the extent to which they have been conserved in Dictyostelium. **Figure 2** shows a scheme of autophagosome formation and conserved proteins that can be identified in the Dictyostelium genome by comparison with the available information in yeast and mammalian systems.

Autophagy induction and its regulation must be tightly controlled by the energy and nutritional status of the cell. The nutrient sensor TOR (target of rapamycin) belongs to a protein family of conserved serine/threonine kinases known as phosphatidylinositol kinase-related kinases. TOR receives a wide variety of intra- and extracellular signals such as nutrients, energy, growth factors, calcium and amino acids.[40,41] TOR associates with different proteins to form two complexes and only one of them, TORC1, is primarily involved in autophagy. The *S. cerevisiae* TORC1 contains Tor1 or 2, Kog1, Tco89 and Lst8 and is sensitive to rapamycin. As in higher eukaryotes, the Dictyostelium genome codes for proteins highly similar to Tor, Kog1 (also known as Raptor) and

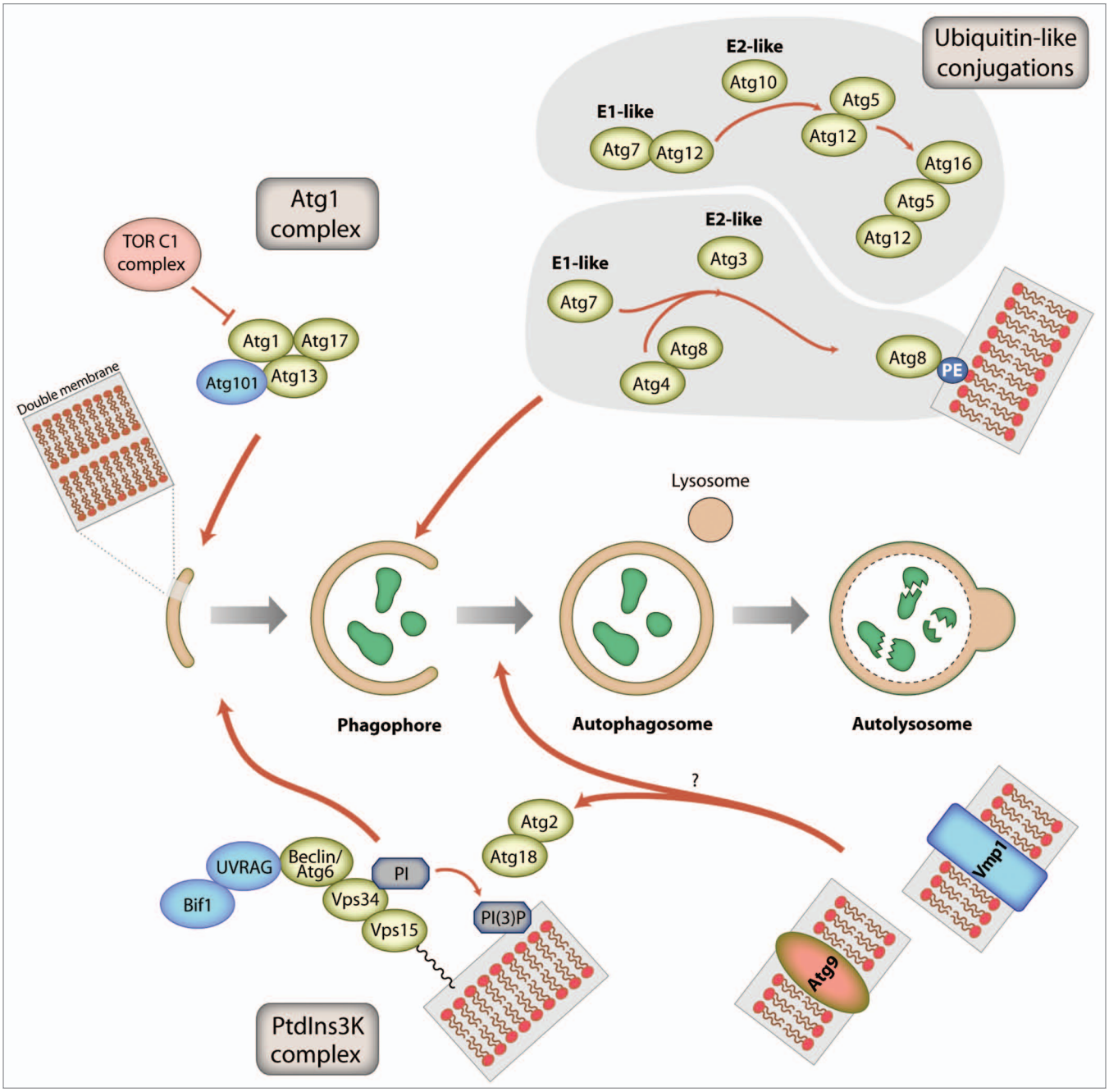

Figure 2. Autophagosome formation and putative signaling pathways in Dictyostelium. The phagophore is a double membrane whose origin is still a matter of debate. This membrane enlarges and finally engulfs parts of the cytoplasm. After fusion with lysosomes the content is degraded and recycled. The predicted Dictyostelium autophagic proteins have been organized in hypothetical functional complexes using the information available from the yeast *S. cerevisiae* and mammalian cells. Some proteins such as Atg101, UVRAG, Bif-1 and Vmp1 seem to be present in Dictyostelium and higher eukaryotes but are absent in *S. cerevisiae*. Vmp1 and Atg9 are transmembrane proteins whose functions are not completely characterized and have been proposed to be involved in membrane trafficking during autophagosome formation.

Lst8. TORC1 regulates many different aspects of cell growth and metabolism and functions upstream of the Atg1 complex, a protein complex containing the kinase Atg1 that plays a central role in the regulation of autophagy by integrating signals from the cellular nutrient status (via its regulation by TOR) and recruiting other autophagy proteins to the site of autophagosome origin (reviewed in ref. 41 and 42). The protein subunit composition of the Atg1 complex and the interplay among these subunits is tightly regulated and depends on TOR activity. Dictyostelium codes for proteins with significant similarity to several Atg1 complex subunits (**Table 1**) and functional analyses have been carried out on Atg1, as described below.

Atg1 is a serine/threonine kinase whose activity is required for autophagy in many different model systems[43-49] including

Table 1. Atg1 protein complex subunits

	Function/features	Dictyostelium	Human	*S. cerevisiae*	E-value Dd-Hu	E-value Dd-Sc
Atg1	Serine/threonine-kinase. (Ma; Cvt)*	Atg1 (DDB_G0292390)	ULK1 (G.ID: 8408) ULK2 (G.ID: 9706)	Atg1 (YGL180W)	1e-38 7e-39	7e-42
Atg13	Atg1 regulator. (Ma; Cvt)	DDB_G0269162	Atg13 related protein (NP_001136145.1)	Atg13 (YPR185W)	n.s.**	n.s.
Atg17	Scaffold protein. (Ma)	Atg17 (DDB0237867)	-	Atg17 (YLR423C)		2e-3
Atg101	Atg1 complex-interacting protein. (Ma)	DDB_G0288287	Atg101 (G.ID: 60673)	-	2e-8	
FIP200	Atg1 complex-interacting protein. (Ma)	DDB_G0268498	FIP200 (G.ID: 9821)	-	1e-3	
Atg29	Atg17-interacting protein. (Ma)	-	-	Atg29 (YPL166W)		
Atg31	Atg17/Atg29-interacting Protein. (Ma)	-	-	Atg31 (YDR022C)		

*Ma, Macroautophagy; Cvt, Cytoplasm-to-vacuole targeting. **The putative Dictyostelium Atg13 shows no significant homology (n.s.) with yeast and human homologues but it contains a conserved pfam Atg13 superfamily domain (as determined by a search of conserved domains at NCBI: http://www.ncbi.nlm.nih.gov/).

Dictyostelium.[16] Dictyostelium Atg1 has an N-terminal kinase domain that shares a high degree of similarity with its counterparts in other organisms, and a poorly conserved C-terminal region. Both domains are separated by an asparagine-rich sequence.[15] The kinase domain is essential for the function of the protein in autophagy, as kinase-dead DdAtg1 has a dominant-negative effect, resulting in a mutant phenotype similar to that observed in the null strain.[16] Interestingly the C terminus contains a short region of significant similarity with human Ulk2 that is also present in other Atg1 proteins such as in Arabidopsis and *C. elegans* but is absent from the *S. cerevisiae* Atg1. The precise function of this domain is unknown, but its presence is required for autophagy.[16]

In *S. cerevisiae,* Atg1 interaction with Atg13 and Atg17 is essential for autophagy induction. This interaction is prevented under nutrient-rich conditions by TOR-dependent phosphorylation of Atg13.[50,51] Starvation conditions inhibit TOR activity and Atg13 becomes rapidly dephosphorylated allowing Atg13 and Atg17 to interact with Atg1 and to activate its kinase activity.[51] Rapamycin, an inhibitor of TOR, is a classic activator of autophagy even under nutrient-rich conditions. A putative Dictyostelium Atg13 homologue has been annotated in the Dictyostelium database (Dicty-base: http://www.dictybase.org/). Although it contains a conserved Atg13 domain that is present in the pfam database (http://pfam.sanger.ac.uk/), Atg13 shows a very low level of similarity between species, suggesting that this protein has largely diverged during evolution.[52]

As seen in **Table 1**, the mammalian Atg1 complex also contains FIP200 (focal adhesion kinase (FAK) family interacting protein of 200 kDa), also known as RB1CC1 (Retinoblastoma 1 inducible coiled coil-1). FIP200 is a multifunctional protein involved in multiple cellular processes besides autophagy such as cell adhesion, migration, cell death and proliferation. It interacts with many different proteins and it is believed to be a functional homologue of Atg17 although they do not share sequence similarity.[53,54] Dictyostelium has putative Atg17 and FIP200, but their level of similarity is too low to decide with some confidence whether or not they are real homologues without any further experimental evidence.

In *S. cerevisiae*, under nutrient-replete growth conditions, Atg1 also regulates the autophagy-dependent cytoplasm-to-vacuole targeting (Cvt) pathway, a mechanism that targets specific hydrolases to the vacuole of *S. cerevisiae*.[55] The hydrolases are packed into autophagosome-like vesicles and delivered to the vacuole in a manner similar to that used during autophagy.[56] This specific and biosynthetic form of autophagy has only been described in *S. cerevisiae* and related yeasts.[57,58] Atg1 and Atg13 are required for both autophagy and the Cvt pathway, but Atg17 is specific to autophagy. A number of other Atg1 complex subunit proteins are known to have specific roles in these pathways. Atg29 and Atg31 are specific for autophagy while Atg11, Atg20 and Atg24 (**Suppl. Table 1**) are only required for the Cvt pathway.[59,60] As in humans, no protein similar to any of these proteins can be recognized by sequence similarity in Dictyostelium (see **Table 1 and Suppl. Table 1**), except for Atg24.

Interestingly, a putative homologue of the mammalian protein Atg101, absent in yeast, can be found in the Dictyostelium genome. Atg101 is a recently described protein essential for autophagy that interacts with Ulk1 in an Atg13-dependent manner. Additionally, it contributes to Atg13 function by protecting Atg13 from proteasomal degradation.[52,61]

Nucleation and the Phosphatidylinositol 3-Kinase (PtdIns3K) Complex

In *S. cerevisiae,* the class III PtdIns3K Vps34 (vacuolar protein sorting 34) is a lipid kinase necessary for autophagy and the Cvt pathway.[62] Its activity generates phosphatidylinositol-3-P (PtdIns3P), believed to be required for binding of other autophagic proteins to the autophagosome nucleation site, such as the

Table 2. PtdIns3K protein complex subunits

	Function/features	Dictyostelium	Human	*S. cerevisiae*	E-value Dd-Hu	E-value Dd-Sc
Atg6/Beclin 1	Subunit of the PtdIns3K complex. (Ma; Cvt)	Atg6B (DDB_G0288021) Atg6A (DDB_G0269244)	BECN1 (G.ID: 8678) BECN1L1 (G.ID: 441925)	Atg6 (YPL120W)	1e-51 2e-27	5e-22 1e-10
Vps34	Class III-phosphatidylinositol 3-kinase. (Ma; Cvt)	PikE (DDB_G0289601) Lower homology to Class I PI3Ks (PikA-H)	PIK3C3 (G.ID: 5289)	Vps34 (YLR240W)	1e-99	1e-121
Vps15	Myristoylated serine/ threonine protein kinase. (Ma; Cvt)	Vps15 (DDB_G0282627) Lower homology at the kinase domain of other proteins	PIK3R4 (G.ID: 30849)	Vps15 (YBR097W)	8e-73	2e-62
UVRAG	Regulates the Beclin1-PtdIns3K complex. (Ma)	DDB_G0288175 DDB_G0283825	UVRAG (G.ID: 7405)	-	1e-28 3e-12	
Bif-1	BAR and SH3-containing protein. (Ma)	DDB_G0284997	SH3GLB1/Bif1 (G.ID: 51100)	-	0.014*	
Atg14	Regulates PtdIns3K. (Ma; Cvt)	DDB_G0278351	KIAA0831	Atg14 (YBR128C)	0.03	n.s.

*The possible Dictyostelium homologue for Bif1 has low homology but contains the expected C-terminal SH3 domain and an N-terminal BAR domain.

phosphoinositide interacting proteins Atg18 and Atg21.[63-65] Besides autophagy, Vps34 is also implicated in other signaling pathways such as the TOR pathway and G-protein signaling to MAPK.[62] Vps34 interacts with Vps15, a myristoylated protein kinase that seems to regulate Vps34.[66,67] This interaction and the kinase domain of Vps15 are necessary for Vps34 activity, although Vps15 does not seem to phosphorylate Vps34 directly.[62,68] A third protein, Atg6 (known as Beclin 1 or Vps30) is also part of the complex.[69] Atg6/Beclin 1 was first identified as a Bcl-2-interacting protein and it is a mammalian tumor suppressor involved in different cancers.[70,71] The complex containing Vps34, Vps15 and Atg6 additionally interacts with two mutually exclusive proteins in *S. cerevisiae*, Vps38 and Atg14. The first one is involved in the Vps pathway and the second one is required for autophagy and the Cvt pathway.

Similar proteins to Vps34, Vps15 and Atg6 can be easily recognized in Dictyostelium and human (**Table 2**). In contrast, Atg14 appears to be present only in close relatives of *S. cerevisiae* and no highly similar proteins can be found in Dictyostelium and higher eukaryotes. However, it should be noted that recently, a distantly related mammalian Atg14 protein has been identified by computational analysis.[72-74] This mammalian Atg14 and UVRAG (UV-radiation resistance-associated gene), another PtdIns3K complex subunit interact with Beclin 1 and Vps34 in a mutually exclusive way. UVRAG has been proposed to be the functional homologue of Vps38 although they do not show significant identity. Therefore, as described in yeast (concerning Atg14 and Vps38), the mammalian cells might also have two different PtdIns3K complexes containing either Atg14 or UVRAG and their mutually exclusive presence might account for the specific functions of this complex in autophagy and other membrane trafficking processes.[73,74] Interestingly, a putative homologue of UVRAG can be detected in the Dictyostelium genome as shown in **Table 2** with a fairly good e-value score. Sequence comparison with Atg14 did not detect any similar protein in Dictyostelium when compared with *S. cerevisiae* Atg14, but identified a protein with a low score when compared with the human Atg14 (**Table 2**).

Besides UVRAG, the mammalian complex might contain additional proteins not identified in yeast such as Ambra1 and Bif-1 whose functions are being characterized.[75,76] Bif-1 interacts with UVRAG and promotes the activation of Vps34. Bif-1 contains two characteristic domains, an amino-terminal N-BAR (Bin-Amphiphysin-Rys) domain, and a carboxy-terminal SH3 (Src-homology 3) and has been proposed to be involved in the biogenesis of the autophagosome membrane due to its membrane binding and bending activities.[77,78] While no similar proteins can be recognized in Dictyostelium for Ambra1, a putative Bif-1 can be identified and, although it shows a low level of similarity, the predicted sequence has the characteristic BAR and SH3 functional domains.

Vesicle Expansion and Ubiquitin-Like Conjugation Systems

Membrane expansion into a fully developed autophagosome requires the function of two ubiquitin-like protein conjugation reactions.[79] In the first conjugation system Atg12 is covalently bound to Atg5, a reaction catalyzed by the E1-type enzyme Atg7 and the E2 enzyme Atg10.[80,81] Atg16 interacts noncovalently with Atg12-Atg5 to form a complex that multimerizes.[82,83] This reaction and the localization of the Atg12-Atg5-Atg16 complex may facilitate the second conjugation reaction, and/or dictate in part where this reaction occurs. In the second reaction the ubiquitin-like protein Atg8 (commonly known as LC3 in mammals) is attached to the expanding autophagosome membrane by conjugation to phosphatidylethanolamine.[84,85] Atg8 is first processed by the protease Atg4 to uncover a conserved glycine at the C terminus that is then used for the covalent binding to the phospholipid with the aid of the E1-type enzyme Atg7, also used in the first conjugation reaction, and the E2-type enzyme Atg3.

The proteins involved in these reactions are very well conserved during evolution and can be easily recognized by sequence similarity in Dictyostelium as shown in **Table 3**. Of note, two Atg8-like proteins are present in Dictyostelium, whereas only one

Table 3. Ubiquitin-like conjugation systems

	Function/features	Dictyostelium	Human	*S. cerevisiae*	E-value Dd-Hu	E-value Dd-Sc
Atg3	E2-like enzyme. (Ma; Cvt)	Atg3 (DDB_G0277319)	Atg3 (G.ID: 64422)	Atg3 (YNR007C)	1e-39	7e-19
Atg4	Cysteine protease. (Ma; Cvt)	Atg4 (DDB_G0273443) DDB_G0283753	Atg4B (G.ID: 23192) Other homologues (Atg4A, C, D)	Atg4 (YNL223W)	3e-19 5e-24	2e-11 1e-12
Atg5	Conjugates with Atg12. (Ma; Cvt)	Atg5 (DDB_G0289881)	Atg5 (G.ID: 9474)	Atg5 (YPL149W)	1e-15	5e-6
Atg7	E1-like enzyme. (Ma; Cvt)	Atg7 (DDB_G0271096)	Atg7 (G.ID: 10533)	Atg7 (YHR171W)	1e-148	1e-116
Atg8	Ubiquitin-like protein that conjugates with phosphatidylethanolamine (PE). (Ma; Cvt)	Atg8 (DDB_G0286191) DDB_G0290491	GABARAP (G.ID: 11337) Other homologues (LC3/ MAP1LC3A; GATE16/ GABARAPL2, etc.,)	Atg8 (YBL078C)	3e-30 2e-21	1e-35 3e-29
Atg10	E2-like enzyme. (Ma; Cvt)	Atg10 (DDB_G0268840)	Atg10 (G.ID: 83734)	Atg10 (YLL042C)	4e-18	0.97
Atg12	Conjugates with Atg5 (Ma; Cvt)	Atg12 (DDB_G0282929)	Atg12 (G.ID: 9140)	Atg12 (YBR217W)	1e-14	8e-7
Atg16	Interaction with Atg12-Atg5 conjugates (Ma; Cvt)	TipD (DDB_G0275323)	Atg16L1 (G.ID: 55054)	Atg16 (YMR159C)	1e-68	1e-4

Table 4. Other autophagic proteins

	Function/features	Dictyostelium	Human	*S. cerevisiae*	E-value Dd-Hu	E-value Dd-Sc
Atg2	Peripheral membrane protein involved in Atg9 cycling (Ma; Cvt)	DDB_G0277419	Atg2A (G.ID: 23130) Atg2B (G.ID: 55102)	Atg2 (YNL242W)	7e-11 4e-24	4e-29
Atg9	Transmembrane protein (Ma; Cvt)	Atg9 (DDB_G0285323)	Atg9A (G.ID: 79065) Atg9B (G.ID: 285973)	Atg9 (YDL149W)	9e-67 1e-26	3e-88
Atg15	Lipase (Ma; Cvt)	-	-	Atg15 (YCR068W)		
Atg18	WD repeat domain phosphoinositide-interacting protein (Ma; Cvt)	Atg18 (DDB_G0285375) Wdr45l (DDB_G0282581)	WIPI-3 (56270) Other homologues (WIPI-1; WIPI-3; WDR45L/WIPI-3)	Atg18 (YFR021W)	1e-37 8e-81	4e-35 8e-29
Atg22	Amino acid export from vacuole (Ma; Cvt)	-	-	Atg22 (YCL038C)		
Atg23	Peripheral membrane protein. (Ma; Cvt)	-	-	Atg23 (YLR431C)		
Atg27	Type I membrane protein. (Ma; Cvt)	-	-	Atg27 (YJL178C)		
Vmp1	Transmembrane protein (Ma)	Vmp1 (DDB_G0285175)	TMEM49 (G.ID: 81671)	-	6e-61	

is present in yeast. Remarkably, the level of similarity between the Dictyostelium and the human proteins is generally higher than that observed between Dictyostelium and *S. cerevisiae* homologues. Another striking similarity is the presence of an extended C terminus in Atg16 containing multiple WD-40 repeats, a feature typically found in the Atg16 homologues of animals but absent in fungi. This domain is probably involved in additional protein-protein interactions that might have been conserved between Dictyostelium and animals. The putative Atg16 homologue (named TipD) was targeted by insertional mutagenesis in a genetic screen for a multi-tipped phenotype but its requirement in autophagy was not addressed.[86] Interestingly the developmental phenotype observed in the *tipD*$^-$ mutant is similar to that described in other Dictyostelium mutants affecting both conjugation reactions, such as *atg7*$^-$, *atg5*$^-$ and *atg8*$^-$.[15,87]

Other Autophagy-Related Proteins

A number of autophagy proteins not included in the above-mentioned functional clusters are involved in other less known processes such as the transport and recycling of components from the autophagosome. As shown in **Table 4**, several of these proteins, such as Atg2, Atg9 and Atg18, can be recognized in the *S. cerevisiae*, Dictyostelium and human genomes. Vmp1 on the other hand is absent in fungi but present in Dictyostelium and higher organisms, another example of the evolutionary proximity of Dictyostelium and animals.

Atg9 is a multispanning membrane protein involved in membrane traffic from not well-defined cellular compartments to the autophagosome and is therefore believed to play a role in the origin and elongation of the autophagic membrane.[88,89] The subcellular localization of Atg9 depends on the organism under study. In *S. cerevisiae,* Atg9 appears to be located on the surface of mitochondria or in vesicles in very close proximity to these organelles.[90,91] In mammalian cells, Atg9 traffics between the Golgi and endosomes suggesting an involvement of the Golgi complex in the autophagic pathway. In Dictyostelium, Atg9 resides in small vesicles that travel from the cell's periphery to the microtubule-organizing center. Its deletion leads to a pleiotropic phenotype including autophagy defects.[92]

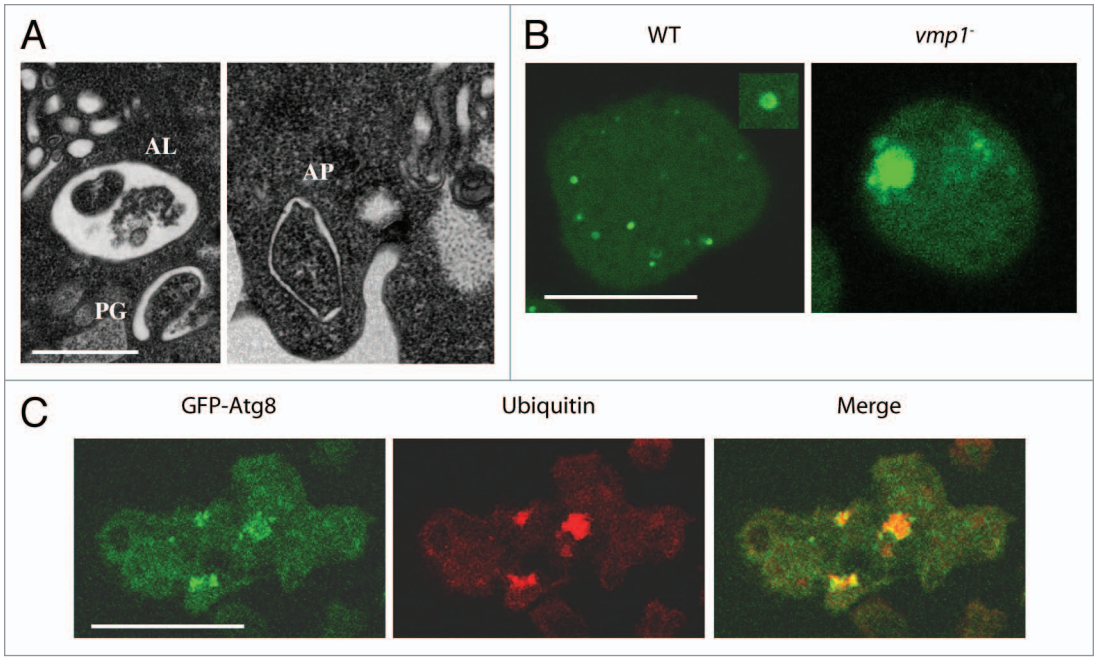

Figure 3. Monitoring autophagy in Dictyostelium. (A) Examples of autophagic structures in Dictyostelium as seen by transmission electron microscopy. PG, AP and AL correspond to putative phagophore, autophagosome and autolysosome respectively. Bar: 0.5 μm. (B) Fluorescence microscopy of wild-type (WT) and *vmp1*- cells expressing the autophagosome marker GFP-Atg8. Close examination of the punctate pattern (see the inset) reveals a vesicle-like appearance of the autophagosomes. In the mutant the marker appears aggregated. Bar: 10 μm. (C) These protein aggregates observed in *vmp1*- organisms (and other autophagic mutants, see the text) colocalize with ubiquitin as determine by immunofluorescence with anti-ubiquitin antibody and contain the scaffold protein p62 as observed in many different protein aggregation diseases.

The fact that Vmp1 and Atg9 are transmembrane proteins known to be required for autophagosome formation in mammalian cells raises interesting questions about their possible role membrane traffic during autophagy.[93-95] In contrast to Atg9, Vmp1 is an endoplasmic reticulum-resident protein in Dictyostelium. It is not yet clear to what extent the autophagosome membrane originates de novo or from pre-existing organelles. The localization of Vmp1 to the ER and its partial colocalization with autophagosomes are in line with other studies that suggest the involvement of the ER in autophagy.[96-99]

Atg18 interacts with Atg2, and this complex is localized to the autophagosome membrane via Atg18 binding to PtdIns(3)P, through the novel conserved motif FRRG.[64,65] This localization is essential for the recruitment of other autophagic proteins and for autophagy, although the precise function of these proteins is unknown.

There are a number of proteins specifically involved in selective autophagy in *S. cerevisiae*. Most of these proteins do not have clear homologues in Dictyostelium and human except for Atg21, a homologue of Atg18 and Atg24, a member of the sorting nexin family with a phosphoinositide binding Phox domain and a BAR domain (**Suppl. Table 1**). This protein is also involved in endosomal protein sorting. The lack of similar proteins is not surprising taking into account that most of these proteins are involved in the Cvt pathway, a process not present in Dictyostelium.

In summary, the Dictyostelium genome codes for most of the basic components that have been described to regulate autophagy. Moreover, the strong similarity with animals and the presence of certain proteins conserved in Dictyostelium and humans that are absent in yeast emphasize the high level of conservation of the basic autophagy machinery between this simple social amoeba and man.

Monitoring Autophagy in Dictyostelium

Transmission electron microscopy (TEM) has been a classical method to monitor autophagy although interpretation of the structures is difficult since autophagosome formation is a very dynamic process with morphologically different stages of maturation. Clear criteria must be applied to determine if a given structure is a bona fide autophagosome, such as the presence of double-membrane vesicles containing organelles or material similar in density to the cytoplasm. This double membrane might have a cup-shape when the formation of the autophagosome has not been completed. When the autophagosome is fused with the lysosomes, the internal membrane and the cytoplasmic material might appear partially degraded. **Figure 3** shows an example of such structures in Dictyostelium. During vegetative growth, TEM images of Dictyostelium cells show very few double membrane autophagosomes, and most of the vacuoles are single membrane and contain spongy material that is believed to correspond to different degrees of digestion of the axenic medium that has been taken up by macropinocytosis.[100,101] Other vesicles are electron-lucent and probably correspond to contractile vacuoles.[100] However, during starvation the number of food vacuoles decreases and double-membrane autophagosomes become

abundant reaching a maximum around 4–5 hours after the initiation of starvation,[100] confirming the activation of autophagy by starvation in Dictyostelium, as described in other organisms. The absence of autophagosomes has been determined by TEM in several Dictyostelium autophagic mutants including *atg1*$^{-}$, *atg6*$^{-}$, *atg8*$^{-}$, *atg7*$^{-}$, *atg5*$^{-}$ *and vmp1*$^{-}$.[15,87,93] Another characteristic feature of TEM images is the progressive disappearance of cytoplasm and organelles during starvation in wild type as a consequence of autophagy. Conversely, the autophagic mutants show dense cytoplasm with little degradation.[15,87]

Molecular markers of autophagy are proteins involved in the autophagy process that can be used to monitor autophagy. The most common marker is Atg8/LC3 that becomes lipidated and attached to the autophagosome membrane, and participates in its elongation. The use of GFP-Atg8/LC3 allows in vivo visualization of autophagy by confocal fluorescence microscopy. In mammalian cells and Dictyostelium, this marker appears as a punctate pattern, as illustrated in **Figure 3**. Since autophagy is a dynamic process involving induction, maturation and degradation, a defect in a particular stage affects the Atg8/LC3 pattern in different ways. For example, a suppression of an early step of autophagosome formation will decrease the number of puncta, but a blockage of late stages might leave the induction unaffected, resulting in an accumulation of puncta.[102,103] In Dictyostelium, the use of the GFP-Atg8 marker reveals some specific features of the system that must be taken into account. Although TEM analysis showed that starvation increases the number of autophagosomes, a number of GFP-Atg8 puncta are present during growth conditions and this number does not seem to be significantly affected during starvation. However, closer examination shows differences in the morphology of puncta. During growth, most of the puncta appear as simple dots. Conversely, during starvation the number of structures showing a cup-like or vesicle-like shape increases (**Fig. 3**). A possible interpretation is that during growth there are many initial autophagosome origins that do not progress in their elongation probably because they require additional signaling events. This signaling would be triggered by starvation to promote the activation of autophagy, and therefore the vesicle-like puncta, reflecting bona fide autophagosomes, become more evident. Alternatively, the dot-like structures observed during growth might represent artifactual aggregation of Atg8/LC3 as described in other systems.[104]

Interestingly, when autophagy is blocked by genetic ablation of Atg1 or Vmp1 in Dictyostelium, the GFP-Atg8 marker colocalizes with large ubiquitinated protein aggregates together with p62 (**Fig. 3**). This phenomenon is less pronounced in other mutants such as Atg7 and Atg5. These aggregates have been described in many other systems where autophagy has been inhibited.[105-107] The accumulation of these aggregates suggests a role for autophagy in their clearance. Other markers that associate with the phagophore have been used in other systems to monitor autophagy, such as Atg5, Atg12, Atg16 and Atg18.[108-110] As described above, Dictyostelium possess proteins highly similar to each of them. They could potentially be used as additional markers to overcome some of the problems observed with GFP-Atg8.

The use of certain substrates to monitor autophagy-dependent protein degradation allows asking whether or not autophagy reaches its last stages, providing information about the autophagic flux. Since Atg8/LC3 and p62 are degraded by autophagy the total amount of these proteins decreases upon autophagy induction despite the expected transcriptional activation. Therefore, the total amount of these markers inversely correlates with autophagic flux.[103,111,112] Dictyostelium cells expressing GFP-Atg8 can be used to monitor the degradation of this marker by western blot using anti-GFP antibodies. As expected we found that the amount of this marker decreases in the first hours of starvation and this decrease is prevented in the autophagic mutant *atg1*$^{-}$ (unpublished observation), suggesting that a similar mechanism operates in Dictyostelium and could be used to monitor autophagy.

The conservation of autophagy genes and the mechanisms involved make us believe that some other techniques used to monitor autophagy in other systems might be applied to Dictyostelium in the future as more research teams join the field and use this model system to study autophagy.

Dictyostelium Autophagy Mutants are Affected in Development

Insertional and knockout mutants have been generated for several Dictyostelium autophagy genes as shown in **Table 5**. They comprise at least one component of each of the described functional complexes: Atg1 from the Atg1 complex,[15] Atg6/Beclin 1 from the PtdIns3K complex,[15] Atg5, Atg7, Atg8 and Atg16[15,86,87] from the ubiquitin-like conjugation systems. Similarly, the two transmembrane proteins identified in mammalian cells to have an essential role in autophagosome formation, Atg9[92] and Vmp1[93,99] have also been ablated in Dictyostelium.

Autophagy is required for multicellular development in Dictyostelium and, interestingly, the severity of the phenotypes depends on the mutated gene. Mutants affected in the ubiquitin-like conjugation systems and Atg6/Beclin 1 have a defect at the mound/finger stage with the formation of multi-tipped structures leading to small or abnormal fruiting bodies.[15,86,87] As described above in **Table 2**, the Dictyostelium genome codes for two homologous Atg6 proteins (Atg6A and Atg6B) and only the first one has been disrupted. As a consequence, the phenotype observed might be affected by partial redundancy.

Stronger phenotypes have been observed in the mutants affecting Atg1 or the transmembrane proteins Atg9 and Vmp1. They show vegetative growth defects, and development is partially or totally arrested at the aggregation or mound stages, depending on the experimental conditions. It should be noted that whereas the proteins involved in ubiquitin-like conjugation reactions seem to play specific roles in autophagy, the Atg1 complex,[42] the PtdIns3K complex,[62] Atg9 and Vmp1[98] might be involved in other membrane trafficking processes. The strong phenotype observed in some of these mutants might therefore be attributed in part to other possible additional defects not directly related to autophagy.

Table 5. Dictyostelium autophagic mutants and related phenotype

Mutant and parental strain	Developmental phenotype	Growth	Survival to starvation	Ubiquitin$^+$ aggregates	References
atg1$^-$ (DH1)	Aggregation/mound arrest	Slow growth	affected	Presence of large aggregates	Otto et al. 2004 (15)
atg5$^-$ (DH1)	Aggregation/Multi-tipped aggregates/ aberrant fruiting bodies	Normal growth	affected	Presence of small aggregates	Otto et al. 2003 (87) Calvo-Garrido and Escalante. 2010 (99)
atg6$^-$ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
atg7$^-$ (DH1)	Aggregation/Multi-tipped aggregates/ aberrant fruiting bodies	Normal growth	affected	Presence of small aggregates	Otto et al. 2003 (87)
atg8$^-$ (DH1)	Multi-tipped aggregates/small fruiting bodies	Normal growth	affected	Not detected	Otto et al. 2004 (15)
atg9$^-$ (AX2)	Aggregation/Multi-tipped aggregates/ aberrant fruiting bodies	Slow growth	Not analyzed	Not analyzed	Tung et al. 2010 (92)
TipD/atg16- (AX4)	Multi-tipped aggregates/small fruiting bodies	Not analyzed	Not analyzed	Not analyzed	Stege at al. 1999 (86)
vmp1$^-$ (AX4)	Aggregation/mound arrest	Slow growth	affected	Presence of large aggregates	Calvo-Garrido and Escalante. 2010 (99)

A similar argument, that autophagy may be required during all stages of the Dictyostelium developmental program, arises from the study of temperature-sensitive Atg1 mutants.[16] Development is arrested when the mutant is shifted to the restrictive temperature even after 16 hours of development when the structures are at the slug stage. Development is then resumed when they are set back to the permissive temperature.[16] It seems that a constant turnover of cellular material might be required at all stages of Dictyostelium development. However, as stated before, since Atg1 has been proposed to play additional roles besides autophagy, the Atg1 requirement during development might also involve other functional aspects that have not yet been characterized.

At the cellular level, dysfunction in protein degradation pathways such as in the ubiquitin-proteasome system and autophagy might lead to the persistence of ubiquitin-positive protein aggregates, a hallmark of many degenerative diseases. Interestingly, Dictyostelium *vmp1$^-$* mutants show accumulation of enormous ubiquitin-positive protein aggregates containing the autophagy marker GFP-Atg8 and the putative Dictyostelium p62 homologue as described in many degenerative human diseases.[99] In mammalian cells, p62 functions as a scaffold protein that provides a link between ubiquitinated aggregates and the autophagy machinery via the direct interaction of p62 with ubiquitin and the autophagosome protein Atg8/LC3. The presence of p62 in these ubiquitinated aggregates suggests that a similar mechanism functions in Dictyostelium. The inability of *vmp1$^-$* cells to clear these aggregates by autophagy would explain their accumulation, as described in mutant mice where the autophagy genes *Atg5* and *Atg9* have been knocked out.[113,114]

The analysis of other Dictyostelium autophagic mutants (*atg1$^-$*, *atg5$^-$*, *atg6$^-$*, *atg7$^-$* and *atg8$^-$*) show a correlation between the severity of their corresponding phenotypes and the presence of ubiquitin-positive protein aggregates.[99] An attractive hypothesis is that the phenotypes are aggravated by the presence of aggregates that might function as a sink for interacting proteins altering their normal localization or concentration. This phenomenon has been recently described in Dictyostelium with the formation of actin inclusions in cells by mistargeting VASP, an actin-binding protein, to endosomes. These actin aggregates are reminiscent of Hirano bodies that are often present in neurodegenerative diseases and, in Dictyostelium, are found to sequester other actin-binding proteins and endosomal proteins, promoting their disappearance from the cytoplasm.[115] These Hirano body-like aggregates can also be induced in Dictyostelium by the overexpression of a truncated form of a 34 kDa actin-binding protein.[116] A recent report shows that both autophagy and the proteasome pathway contribute to the degradation of Hirano bodies in Dictyostelium. Moreover, the autophagosome marker protein GFP-Atg8 colocalizes with model Hirano bodies in wild-type Dictyostelium cells, but not in *atg5$^-$* or *atg1$^-$* cells.[117]

Dictyostelium Autophagic Cell Death

Cell death with autophagy has been observed in particular in development and in pathology.[118] Importantly, in recent years a causative role for autophagy in cell death could be demonstrated in certain cases through the decrease of cell death upon inactivation of an autophagy gene, often with no accompanying causative apoptotic or necrotic cell death.[119-132] The question, then, becomes not whether autophagy is causative in some cases of animal cell death (it clearly can be), but how.

The protist Dictyostelium shows, when starved, developmental formation of a fruiting body consisting of viable spores and dead stalk cells.[133] Stalk cell formation can be mimicked in vitro under monolayer culture conditions, where Dictyostelium cells can differentiate from vegetative into "stalk" vacuolated cells[134-136] showing signs of autophagy (see below) and undergoing cell death. This monolayer model shows many advantages for the study of autophagic cell death (ACD)[33] including the absence of

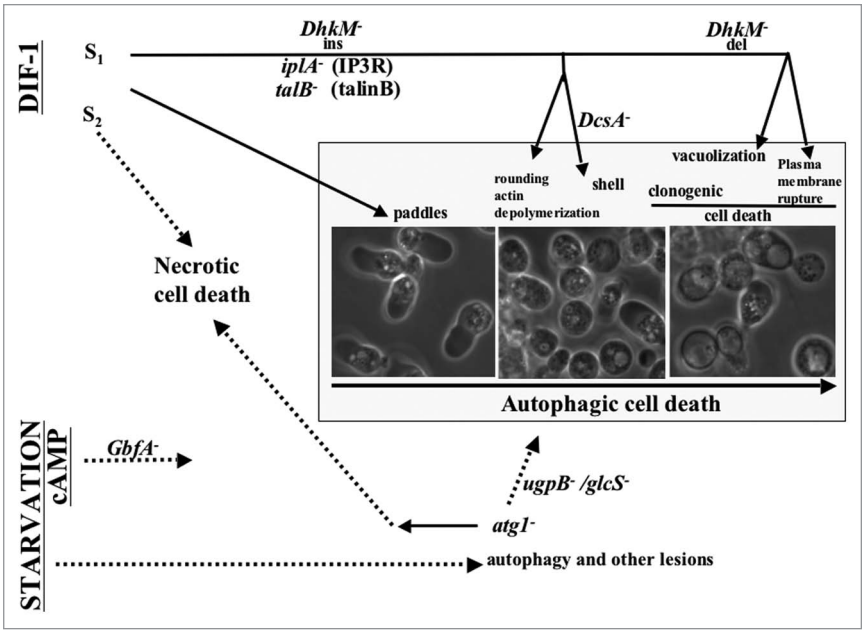

Figure 4. Pathways to cell death in Dictyostelium and mutational analysis thereof. In a first stage (lower half), starvation and cAMP lead to autophagy and sensitize cells to induction by DIF-1 (upper half) of either autophagic (right part) or necrotic (left part) cell death depending on whether the *atg1* gene is wild type or inactivated by mutation. The indicated other mutations allowed the dissection of particular autophagic cell death (see main text).

the main members of the apoptosis machinery that could interfere with it.[137,138]

Importantly, triggering ACD in monolayers requires at least two distinct stimuli. The first stimulus is starvation together with cAMP.[135,139,140] These induce, on the one hand, autophagy, as manifested by the appearance of autophagosomes,[136,141,142] and on the other hand, major mitochondrial lesions.[143] Starvation, cAMP and the resulting alterations including autophagy do not by themselves lead to ACD. To induce cell death, a second stimulus is required, namely the main stalk differentiation-inducing factor DIF-1, a small dichlorinated molecule.[144-147] DIF-1 is naturally synthesized during starvation-induced development. In monolayer experiments, ACD can be induced by addition of cAMP and DIF-1 to starved cells undergoing autophagy.[134-136] ACD includes first the emergence of polarized "paddle" cells, then their rounding and acquisition of a cellulose shell. Small vacuoles then fuse to form large vacuoles ultimately occupying most of the cell volume (**Fig.** 4). Plasma membrane rupture occurs later (50% at around 40 hours of treatment) as judged by propidium iodide staining.[134,136] The whole sequence of ACD subcellular events is shown in **Figure** 4.

ACD has been further investigated in this model, mostly using random insertional mutagenesis,[33] and here its requirements and genetic control are briefly reviewed (summarized in **Fig.** 4). Starvation-induced events are blocked by mutations of *gbfA* (G-box binding factor; a transcription factor)[33] and of *ugpB* (UDP-glucose pyrophosphorylase)/*glcS* (glycogen synthase).[141] The DIF-1-triggered pathway leading to ACD was also studied by insertional mutagenesis. The correct functioning of this pathway from its triggering by DIF-1 to the induction of ACD requires the following genes: *iplA* (IP3 receptor),[148] *talB* (talin B),[33] and *DhkM* (receptor histidine kinase M).[149] Mutation of these genes dissociates the autophagic cell death phenotype into several subcellular traits under various molecular controls.[33,149]

In this system, is autophagy itself mechanistically required for or only accompanying ACD? If autophagy were required for ACD in the Dictyostelium model, mutation of one of the *atg* genes essential for the autophagic pathway should prevent not only autophagy, but also most or all of the signs of ACD. Indeed, an *atg1*$^{-}$ mutation decreases autophagy[15,150] and suppresses ACD.[150] Cell death, however, still occurs upon addition of DIF-1 to starved *atg1*$^{-}$ cells, but as necrotic cell death (NCD), quite distinct from ACD. NCD involves immediate and massive mitochondrial uncoupling, perinuclear clustering of mitochondria, lysosomal permeabilization and rapid plasma membrane rupture.[150-152] Several mutations that inhibit the pathway leading to ACD do not affect or affect much less, the pathway leading to NCD,[33,141,148] and NCD and ACD differ as to the specificity of their DIF-1 signaling.[153] These data are compatible with the interpretation that a mutation of the autophagy gene *atg1* could at the same time affect two distinct types of cell death, leading to NCD and preventing ACD. However, in this model NCD occurs much sooner and quicker than ACD and may thus preempt the occurrence of ACD, leading to the alternative interpretation that the *atg1*$^{-}$ mutation would just favor the more rapid NCD, without any significance as to an Atg1 requirement for ACD. Current investigations aiming at rigorously checking an Atg1 requirement for ACD are based on drastic suppression of NCD. A first approach includes the differential reversibility of ACD and NCD upon early removal of the inducer. Specifically, removal of DIF-1 15 min after its addition led, in *atg1*$^{-}$ cells to full reversal of early signs of NCD and ultimately to no or little death, but in wild-type cells to almost no reversal of ACD, which proceeded to vacuolization and death (reviewed in ref. 136, 151). A second approach is to use as a death-inducer not DIF-1, but a given DIF-1 derivative called 107 or desmethyl-DIF-1, which induces ACD, but almost no NCD.[153] Preliminary experiments using early removal of 107, or yet other approaches, or combinations of these, to prevent induction and/or completion of NCD strongly suggest that ACD is indeed dependent on Atg1 in this model.

Altogether, in this Dictyostelium monolayer model, autophagic cell death triggering requires a first signal, starvation/cAMP, leading to autophagy and a second signal, DIF-1, leading from autophagy to ACD. Autophagy is not directly causative of death (since autophagy is not sufficient) but primes for a mechanism that is (the DIF-1 pathway to ACD can occur only if *atg1* is intact). We think that such a second signal or something homologous to it, may well exist to trigger ACD in less simple eukaryotes,

where it is still buried in complexity. While we do not know yet to what extent this mechanism is conserved for instance in mammalian cells, in some cases the latter can show vacuolizing ACD morphologically very similar to that seen in Dictyostelium.[154] On the pathway triggered by DIF-1, some mutations specifically affect ACD, not autophagy. These mutations dissociate ACD into distinct, separately controlled subcellular lesions. To pursue this genetic analysis of ACD in this very favorable model, a search for further ACD mutants is ongoing.

Autophagy and Infection in Dictyostelium

The first line of defense against invading bacteria is comprised of phagocytic cells of the innate immune system. These cells are specialized in the recognition of invading pathogens and respond by activating antimicrobial immune responses (reviewed in ref. 155). These cells recognize and contain microbes early during infection via complement activation, phagocytosis, autophagy and immune activation by families of pattern recognition receptors (PRRs). The response relies on recognition of evolutionarily conserved structures of commensals and pathogens, termed pathogen-associated molecular patterns (PAMPs). The family of TLRs is the major and most extensively studied class of PRRs. The main bactericidal strategy relies on phagocytosis, the process by which cells engulf particles, which is conserved during evolution. In organisms such as amoebae, "phagotrophy" is used for feeding and appears as a distinguishing feature in the last common ancestor of eukaryotes.[156] In immune phagocytes, the bactericidal and degradation machineries have been harnessed to meet the needs for presentation of antigenic peptides.

Studies of autophagy identified important functions in the regulation of innate immunity and inflammation (reviewed in ref. 157). Xenophagy refers to the use of the autophagy pathway to digest foreign rather than self-constituents. The PRR-triggered pathways and the autophagy process intersect at many different levels: TLRs can regulate autophagy induction, the autophagy machinery can be used to deliver pathogen genetic material for binding to endosomal TLRs, and TLRs may act in the recruitment of autophagy proteins to phagosomal membranes. Indeed, Atg proteins have been identified in the major proteomic investigations of phagosomal components.[158] The pathways leading from bacterial sensing to xenophagy are very complex and have not been completely deciphered yet. Nevertheless, a picture is emerging with a central axis of signaling making use of the general nutrient-sensing cascade involving the energy sensor AMP-activated protein kinase (AMPK) that, in response to high AMP/ATP ratios, inhibits TORC1 and leads to induction of autophagy (reviewed in ref. 159). In addition, during evolution, before the NFkappaB pathway emerged as the central coordinator of the immune response, the p38 mitogen-activated protein kinase (MAPK) cascade served as the ancestral antimicrobial defense-coordinating pathway.[160]

Facing the evolution of ever more efficient bacterial sensing and killing mechanisms, microorganisms subject to predation were under strong selective pressure to develop the traits needed to survive phagocytic cells, including passive (resistant capsule) or active (toxin secretion) defense mechanisms, but also the ability to replicate directly within the predator cell. This results in a paradox: many microorganisms, although they only accidentally infect mammals, have evolved sophisticated mechanisms to do so.[161] One of the clearest examples is Legionella that did not infect humans before the invention of air conditioning. Indeed, the virulence traits of Legionella and pathogens such as Chlamydia and waterborne Mycobacteria[162] were probably selected to fight amoebae long before the appearance of metazoans. Despite evolutionary perfection, phagocytic cells can be hijacked by intracellular pathogens that overcome their killing mechanisms and establish themselves a vacuolar or cytosolic niche to survive and/or proliferate. Upon cell invasion, bacteria must confront xenophagy, an efficient intracellular defense machinery. Beside bacteria that are completely controlled by autophagy as part of the innate surveillance mechanism, several bacterial pathogens have evolved virulence strategies to either inhibit autophagy to establish a persistent infection or even to take advantage of autophagy to generate a replication niche and to succeed in colonization and spreading (reviewed in ref. 163).

The amoeba Dictyostelium is an attractive model system to study host-pathogen interactions.[25,164] Recent reports suggest that self-nonself discrimination[165] and innate immunity[166] already evolved in amoebae. Dictyostelium cells feed on soil bacteria and, throughout their life, ingest, kill and digest microorganisms at a rate of at least one per minute. Thus, Dictyostelium is likely to have evolved mechanisms that enable it to discriminate and respond appropriately to various bacteria to optimize feeding and to avoid subversion by pathogens. Indeed, genome-wide mutagenesis screening reveals pathways of uptake and killing mechanisms specific to $Gram^+$ or $Gram^-$ bacteria.[167] Several transcriptomic analyses of Dictyostelium's reaction to different bacterial species have been carried out and reveal strong modulation of thousands of transcripts.[168-170] Many of these genes belong to a set of "innate immunity-related" genes that bear homologies to plant and insect innate immune defenses, as well as to the mammalian pathways,[27,168] confirming that Dictyostelium can recognize bacteria and modulate its response.[26] In the multicellular slug, a special cell-type, the sentinel cell, patrols in search of xenobiotics and bacteria.[166]

Because of its ease of manipulation and the conservation of cell-autonomous defense pathways, Dictyostelium has been successfully used and instrumental in the study of virulence mechanisms of Pseudomonas, Legionella and *Vibrio cholera*.[171-174] Most interesting in the view of autophagy, Dictyostelium is an experimental host to pathogens that interact and interfere with xenophagy such as Salmonella, mycobacteria and especially Legionella.[175]

Salmonella enterica serovar Typhimurium is a food-borne pathogen that is usually restricted to the gastrointestinal tract, but can cause severe extra-intestinal diseases in the elderly. In epithelial and other cell types, Salmonella escapes the phagosome pathway and establishes a replication compartment that retains some characteristics of the endosomal pathway. Contrary to the fate of many intracellular pathogens for which the course of infection in Dictyostelium is similar to the one in macrophages, Salmonella is killed and degraded hours after ingestion by the

amoeba.[176] Interestingly, Salmonella appears to evade the common fate of nonpathogenic bacteria such as *E. coli* and escapes phagosome maturation. But, even though Salmonella does not succumb to the bactericidal activities of the phagosomal pathway, it is nevertheless surrounded by GFP-Atg8-positive membranes about 2 hours post-infection and finally is degraded in autolysosomes.[177] Confirming the restrictive role of autophagy, infection of *atg1*-, *atg6*- and *atg7*-null mutants results in the formation of a standard Salmonella-containing vacuole (SCV) and bacteria proliferation. This is finally detrimental to these autophagy-defective Dictyostelium mutants, which die within 1–3 days of infection.[177]

Like many other bacterial pathogens, *M. tuberculosis* can reside in various compartments of its host. As a facultative intracellular pathogen, it can reside outside cells, in the interstitial space or inside necrotic granulomatous lesions. After uptake by immune phagocytes and inducing an arrest of their phagocytic maturation pathway, it resides intracellularly, first inside a replication vacuole[178] and then in the cytosol.[179] In Dictyostelium, the establishment and course of an infection by *M. marinum* are similar to those observed for pathogenic mycobacteria in other host systems. Importantly, as is the case in animal macrophages, during infection of Dictyostelium, *M. marinum* escapes its vacuole and continues to proliferate in the cytosol.[180] It is worth noting that, in activated macrophages, autophagy appears to be able to overcome the phagosome maturation block imposed by mycobacteria and thus controls *M. bovis* BCG infection by directing the replication vacuole to fuse with lysosomes and kill the bacteria.[181] Whether this might also be relevant for infections by *M. marinum* and *M. tuberculosis* still awaits further studies. However, recent studies point to a causality link between vacuole rupture, *M. marinum* exposure to the cytosol, ubiquitination and the spatial recruitment of Atg8-positive membranes, indicating the intervention of adapter proteins such as p62/sequestosome 1. Interestingly, for some cytosolic pathogens, the cell wall is a target for ubiquitination,[182] whereas for others, the damaged vacuole is the target.[183] Furthermore, it is suggested that bactericidal peptides derived from ubiquitin and ribosomal proteins are brought in contact with the mycobacteria via p62-mediated autophagy.[184] Because most of these proteins and processes are conserved in Dictyostelium, including p62,[99] it will be exciting to investigate whether these mechanisms are also active during infection of Dictyostelium by *M. marinum*.

Legionella pneumophila is the prototype of an accidental pathogen for human, because its natural hosts are unicellular protozoa, such as Acanthamoeba. This explains why the use of the amoeba Dictyostelium to study the mechanisms of Legionella virulence and host resistance has been increasingly popular, and represents the "flagship" of host-pathogen studies in this model system. The many successes in this field of research have been very recently and comprehensively reviewed (see ref. 175), and here we will concentrate on the interactions of Legionella with autophagy. Studies in macrophages, mainly using pharmacological tools, had pointed to a potential positive involvement of autophagy in the biogenesis of the replication compartment.[185] For example, starvation-induced autophagy had a modest stimulatory effect on proliferation.[186] But this claim remained disputed, until a seminal study using the genetic power of Dictyostelium demonstrated that the absence of either Atg1, Atg5, Atg6, Atg7 or Atg8 had little or no impact on the establishment of the replication compartment, and even slightly enhanced the proliferation of Legionella.[187] These findings were compatible with a role of autophagy in the control of Legionella infection, but this was not further examined until a few recent studies. The starting point was the finding that the global transcriptomic response to Legionella infection includes the prominent regulation of three autophagy genes encoding Atg8, Atg9 and Atg16.[172] Among these, the multi-transmembrane protein Atg9 was chosen to study the impact of gene ablation in Legionella infection.[92] First, surprisingly, the absence of Atg9 results in a significant decrease in phagocytic uptake, possibly reflecting a direct or indirect coupling between phagocytosis and autophagy. Then, a careful quantitative analysis of the early phase of infection reveals that, in wild-type Dictyostelium, Legionella is rapidly and strongly cleared from the amoeba in the first hours post uptake, and that this is strongly defective in *atg9* null cells.[92] These findings confirm and extend the previous conclusions that autophagy plays a protective role to limit infection by Legionella.

But three recent studies indicate that the case is probably not definitively closed and that the interaction of Legionella with the autophagic pathway might be more complex than initially thought. First, it was recently discovered that an effector secreted by Legionella, AnkB, represents a case of molecular mimicry by which Legionella subverts the polyubiquitination machinery of its host, be it a macrophage or a Dictyostelium cell.[188] This protein contains a noncanonical F-box domain, the integrity of which is essential for rapid acquisition of polyubiquitinated proteins by the Legionella-containing vacuole and for bacteria proliferation. AnkB is proposed to act via a pathway including the SCF1 (RBX1-CUL1-SKP1) ubiquitin ligase complex that is highly conserved throughout eukaryotes.[188] Second, while studying the causes of increased susceptibility of patients with mitochondrial diseases to Legionella infection, Paul Fisher's group highlighted the profound impact of an upregulation of the energy-sensing protein kinase AMPK.[189] Upregulation of AMPK is a primary response to the impaired energy production in such diseases, but the resulting dysfunction on the containment of Legionella infection was a relative surprise. Overexpression of AMPK in wild-type Dictyostelium phenocopied the situation in mutant cells, identifying AMPK as a dominant regulator of intracellular immunity to Legionella,[189] possibly via the TOR-autophagy or p38ERK-MAPK cascade pathways. More work is required to answer these exciting developments, but another study might point in that direction. High-throughput screening to identify host proteins that modulate Legionella growth in Dictyostelium reveal a pivotal role for DupA in the genesis of the replication niche.[169] DupA is a putative tyrosine/dual specificity phosphatase that appears to regulate ERK1 phosphorylation and activation of the MAPK cascade. Also of interest is the finding that many genes are regulated both in *dupA* null cells and upon infection with bacteria, including the *tirA* and *slrA* genes that encode proteins suggested to play an immune-like function in sentinel cells during development.[166]

Concluding Remarks

Autophagy is a fast emerging field and although a big leap has been taken recently by identifying a group of proteins involved in the mechanism and regulation of autophagy, the molecular function of many of these Atg proteins is still poorly defined. It is very likely that a number of proteins involved in autophagy are still unknown and the use of simple experimental models should help us define these new components. Autophagy in the social amoeba Dictyostelium plays essential roles in its natural life that makes it a suitable model where autophagy can be studied in the context of a whole organism. The differences between Dictyostelium and the yeast model *S. cerevisiae* will enrich the possibilities of study while still maintaining the simplicity of the microorganisms. Its powerful molecular genetics, the availability of the genome sequence and the similarities with higher organisms will help shed light on many of the still unanswered questions and help discover new genes involved in this exciting field.

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Note

Supplementry materials can be found at:
www.landesbioscience.com/supplement/CalvoGarridoAUTO6-6-Sup.pdf

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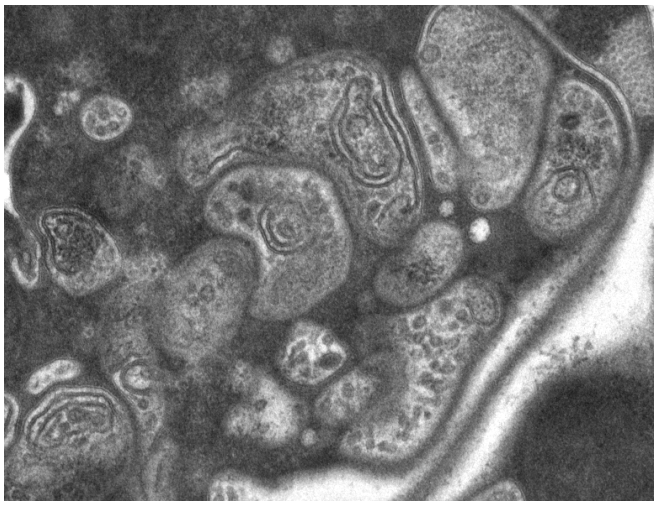

DISCUSIÓN

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Dictyostelium discoideum, un nuevo modelo en autofagia

La enfermedad de Alzheimer (también demencia senil de tipo Alzheimer), la enfermedad de Parkinson (también parálisis agitante) o la enfermedad de Huntington (también corea de Huntington) son enfermedades neurodegenerativas que cursan entre otros procesos con un progresivo deterioro de la función cognitiva y con una amplia degeneración de diferentes zonas cerebrales. La enfermedad de Alzheimer y de Parkinson son cada vez más recurrentes debido al envejecimiento constante y progresivo de la actual población (Banerjee et al. 2010). Las 3 poseen una característica en común, la formación de agregados proteicos en neuronas que parece pueden imposibilitar su correcto funcionamiento y terminar muriendo, aunque también se cree que estos agregados representan una respuesta neuronal a la incapacidad de degradar proteínas mal plegadas, siendo un mecanismo de protección que aparta o retira estas proteínas mal plegadas para permitir un correcto funcionamiento celular. La composición de estos agregados es muy variada, destacando la presencia de proteínas Aβ y tau en la enfermedad de Alzheimer, α-sinucleína en la enfermedad de Parkinson y huntingtina en la enfermedad de Huntington (Ventruti and Cuervo 2007).
Estos agregados proteicos no son exclusivos de patologías en eucariotas superiores, sino que también aparecen en otros organismos modelo más sencillos como *Drosophila melanogaster* (Bartlett et al. 2011), *C.elegans* (Tian et al. 2010), y como se muestra en el capitulo 3 también en *Dictyostelium* ante una disfunción autofágica (Calvo-Garrido and Escalante 2010).

Existen cepas de *Dictyostelium* mutantes en *vmp1*, *atg1*, *atg5*, *atg7*, que muestran agregados proteicos. El tamaño de éstos es proporcional a la severidad del fenotipo que presentan estas cepas en crecimiento y desarrollo, siendo las cepas mutantes de *vmp1* y *atg1* las más severas (Calvo-Garrido and Escalante 2010).

Mediante un sencillo aislamiento hemos conseguido analizar de manera preliminar la composición de estos agregados proteicos en la cepa mutante de *vmp1* en *Dictyostelium*, encontrando ubiquitina, proteínas relacionadas con la ubiquitinación y y el homólogo a la proteína humana p62. P62 es una proteína que funciona como

adaptador o receptor de diferentes cargos autofágicos facilitando su entrada en el autofagosoma, ella misma también es degradada junto con el cargo y por ello su acumulación es muy característica en enfermedades neurodegenerativas usándose además como un marcador de disfunción autofágica.

La mayoría de la información que tenemos del funcionamiento de la autofagia proviene de los estudios realizados en la levadura *Saccharomyces cerevisiae* (Klionsky 2005), sin embargo el funcionamiento de la autofagia en mamíferos presenta muchas más similitudes con *Dictyostelium* que las que presenta con *Saccharomyces cerevisiae*. En mamíferos existen múltiples focos de inicio autofágico al igual que en *Dictyostelium*, mientras que en levaduras encontramos un único foco conocido como sitio pre-autofagosómico. En mamíferos y *Dictyostelium* no existe la presencia de una ruta compartida de síntesis y degradación autofágica como ocurre en *Saccharomyces cerevisiae* con la ruta Cvt. Existen además proteínas relacionadas con autofagia como Vmp1, Atg101 y p62 no presentes en levaduras y sí en mamíferos y *Dictyostelium*.

Si a esto unimos el resto de avances realizados en el campo de la autofagia en *Dictyostelium* (capítulos 3 y 4) como la ampliación del conocimiento acerca del funcionamiento de la proteína fusión GFP-Atg8 en presencia de alimento y ayuno, los estudios de microscopía electrónica, los ensayos de formación de células tallo, los estudios de supervivencia en medios de ayuno, la realización de un estudio comparativo bioinformático del proteoma autofágico de *Dictyostelium*, *Saccharomyces cerevisiae* y humanos y la puesta apunto de un nuevo método de medición del flujo autofágico (datos no mostrados) podemos concluir que *Dictyostelium discoideum* reune muchas de las características necesarias para estudiar este proceso así como el papel de nuevas proteínas como Vmp1 y Atg101. Pensamos que es posible extrapolar este conocimiento a eucariotas superiores y de ese modo ayudar a enteder la relación entre la autofagia y los procesos patológicos que puedan derivar de una disfunción de este proceso.

Vmp1, el reto de adentrarse en el estudio de una proteína de función desconocida

El estudio de una proteína de función desconocida presenta grandes dificultades. Existen una serie de puntos clave como son su localización subcelular , sus posibles interactores, o los fenotipos asociados a su cepa mutante o interferida que deben abordarse en profundidad para alcanzar una posible hipótesis sobre su función.

Los abordajes bioinformáticos son inicialmente la mejor herramienta a la hora de poder de encontrar un punto de inicio que permita orientarnos correctamente en la investigación. La información sobre la localización, la presencia de motivos funcionales, la homología con otras proteínas en otras especies, la concurrencia filogenética, la estructura tridimensional y por supuesto el análisis de la bibliografía existente son imprescindibles.

Vmp1 es en *Dictyostelium* una proteína transmembrana con 403 aminoácidos, con una secuencia de retención de retículo endoplásmico KKXX en su región C-terminal y sin dominios funcionales conocidos. El número de regiones transmembrana varía entre 6 y 8 dependiendo del programa bioinformático utilizado. El programa PSORT nos indica con un 66,7% de probabilidad que Vmp1 en *Dictyostelium* es una proteína localizada en el retículo endoplásmico. Existen homólogos a la proteína Vmp1 de *Dictyostelium* en mamíferos, *Drosophila melanogaster*, *C.elegans* entre otros, pero no en levaduras modelo como *Saccharomyces cerevisiae* o *Schizosaccharomyces pombe* (Calvo-Garrido et al. 2008b). Los análisis de concurrencia filogenética con el programa string o la búsqueda de estructuras tridimensionales conservadas en hhpred no depararon ningún resultado informativo.

La bibliografía presente en el inicio de nuestro estudio era muy escasa y tan sólo se había descrito mediante estudios de microarrays la desregulación de la expresión de *vmp1* en procesos cancerígenos (Higgins et al. 2003), su sobreexpresión en la enfermedad de pancreatitis aguda en modelos murinos y que esta sobreexpresión en células cos7 inducía vacuolización citoplásmica y muerte celular (Dusetti et al. 2002, Vaccaro et al. 2003). Con objeto de obtener alguna pista sobre la función molecular de Vmp1 se realizaron diversos abordajes como los que se muestran a continuación.
La búsqueda de posibles interactores de Vmp1 en *Dictyostelium discoideum* se abordó mediante las técnicas de dos híbridos y TAP-TAG (“tandem affinity purification”)

(Meima, Weening and Schaap 2007). La técnica de dos híbridos, requiere que las proteínas sintetizadas en la levadura se desplacen hasta el núcleo. Si tratamos de realizar este abordaje con proteínas de membrana como Vmp1 nos encontraremos con proteínas fuera de su entorno hidrofóbico y raramente podrán plegarse adecuadamente imposibilitando cualquier interacción proteína-proteína. Por ello se realizó un estudio bioinformático previo con el fin de predecir las posibles zonas transmembrana y no transmebrana de la proteína Vmp1. Las zonas no transmembrana que previsiblemente tendrían una probabilidad más alta de plegarse correctamente y ser solubles fueron clonadas y analizadas en búsqueda de interactores.

Por otro lado se intentó la purificación y búsqueda de interactores mediante TAP-TAG de la proteína completa Vmp1-GFP-TAP y de las zonas no transmembrana que habían sido seleccionadas previamente para dos híbridos fusionadas a TAP. El análisis de estas posibles zonas no transmembrana se debe a que la purificación de proteínas de membrana es muy compleja. Al igual que en dos híbridos el mantenimiento de un entorno hidrofóbico similar al que estas proteínas encuentran en la membrana es fundamental para que mantengan una correcta estructura plegada y que sus posibles interacciones con otras proteínas se mantengan. Ninguno de los 3 abordajes deparó resultados positivos, lo cual impidió que pudiesemos reevaluar el fenotipo de la cepa mutante de *vmp1* y tener una visión molecular más amplia de su posible función.

Se realizó también un abordaje genético a gran escala para intentar hallar posibles proteínas reguladoras relacionadas con Vmp1 que complementasen la cepa mutante de *vmp1* mediante el uso de una genoteca de sobreexpresión de cDNA (Robinson and Spudich 2000). Como se muestra anteriormente en los capítulos 1 y 4, células mutantes de *vmp1* no son capaces de formar cuerpos fructíferos en placas bacterianas y además mueren en ayuno a los 3 días. Sin embargo las células “wild type” permanecen vivas hasta el quinto día y son perfectamente capaces de formar cuerpos fructíferos en placas bacterianas. Al transformar células mutantes de *vmp1* con esta genoteca intentábamos obtener transformantes que sobreviviesen 5 días en ayuno y que al ser plaqueados en bacterias formasen cuerpos fructíferos. El único cDNA que complementó la cepa mutante de *vmp1* fue el propio *vmp1*, lo que validaba el planteamiento experimental pero no nos revelaba ningún supresor de la mutación.

Todos estos esfuerzos ponen de relieve la dificultad para establecer la función molecular de una proteína. Existen en la literatura muchos ejemplos, como el de las proteínas α-sinucleína o huntingtina cuya función no está todavía perfectamente aclarada a pesar de su enorme relevancia patológica y el amplio estudio que se les ha dedicado (Clayton and George 1999, Zuchner and Brundin 2008).

La localización subcelular de Vmp1 varía en diferentes tipos celulares

La localización subcelular de Vmp1 es un aspecto de enorme interés y discusión. La localización en *Dictyostelium discoideum* es clara y precisa como se muestra en los capítulos 1 y 3, sin embargo existe controversia en mamíferos donde los datos publicados por otros autores parecen depender del tipo celular y de las condiciones experimentales.

La ausencia de un anticuerpo comercial que reconozca Vmp1 en *Dictyostelium* nos condujo a la construcción de la proteína de fusión Vmp1-GFP-TAP. Esta construcción complementa los fenotipos analizados de la cepa mutante de *vmp1* por lo cual deducimos que la proteína es funcional y su localización correcta. El análisis de localización de Vmp1-GFP-TAP deparó una colocalización completa con el marcador de retículo endoplásmico PDI y parcial con el marcador autofágico Atg8. Ambas localizaciones son apoyadas por algunos de los datos de localización procedentes de mamíferos y por los fenotipos de la cepa mutante de *vmp1* en *Dictyostelium* que muestra tanto defectos en diversos procesos relacionados con trafico de membranas cuyo origen es el retículo endoplásmico como aquellos relacionados con autofagia. Además como anteriormente se indicó las predicciones bioinformáticas reconocían a Vmp1 con un 66,7% de probabilidad como una proteína de retículo endoplásmico (Calvo-Garrido et al. 2008b).

Es importante poner de relieve que la localización de Vmp1 tanto en retículo endoplásmico como en autofagosomas puede tener un sentido funcional ya que el retículo puede actuar como fuente de membranas para la formación de autofagosomas (Tooze and Yoshimori 2010) o por el contrario puede deberse simplemente a un proceso de reticulofagia, donde fragmentos del retículo forman parte

del cargo autofágico (Bernales et al. 2007). El aislamiento y análisis por western blot e inmunocitoquímica de vacuolas autofágicas nos mostraría si Vmp1 realmente está presente en vacuolas autofágicas maduras o simplemente es una proteína de retículo endoplásmico involucrada en la formación inicial del autofagosoma.

Los estudios de otros autores en mamíferos han deparado resultados muy dispares ya que mismos abordajes en líneas celulares como Hela, HEK293, MEF, cos7 o SH-SY5Y han mostrado resultados ampliamente diferentes.

Inicialmente se localizó en células cos7 la proteína de fusión Vmp1-EGFP en vacuolas citoplásmicas además de en zonas compatibles con el retículo endoplásmico y el aparato de Golgi. La formación de estas vacuolas citoplásmicas era provocadas por la propia sobreexpresión de Vmp1, que además inducía muerte celular (Dusetti et al. 2002). Esta localización coincidía con nuestra localización en *Dictyostelium discoideum* de retículo endoplásmico, sin embargo la sobreexpresión de Vmp1-GFP-TAP nunca indujo vacuolización ni muerte celular (Calvo-Garrido et al. 2008b). Otros grupos mostraron posteriormente en líneas celulares de mamífero que la sobreexpresión de Vmp1-GFP no inducía muerte celular (Itakura and Mizushima 2010). Este dato contradictorio se analizará más adelante en profundidad.

Estudios que relacionaron Vmp1 con cáncer mostraron como la proteína de fusión Vmp1-GFP en células HEK293 localiza tanto en retículo endoplásmico, con una clara colocalización con calnexina (proteína presente en retículo endoplásmico) como en membrana plasmática con ZO-1 (“zonula occludens 1”) (Sauermann et al. 2008). Esta diferente localización era debida a los niveles de expresión de la proteína de fusión; ante expresiones más bajas la proteína localizaba en membrana plasmática y expresiones más altas la retenían en retículo endoplásmico. Estudios posteriores con un anticuerpo contra Vmp1 volvieron a mostrar una localización en membrana plasmática al colocalizar con ZO-1, este dato fue confirmado por la coinmunoprecipitación de Vmp1 y ZO-1 (Sauermann et al. 2008). Nuestros estudios nunca encontraron a Vmp1 como una proteína de membrana ni tampoco establecieron ningún fenotipo asociado a membrana plasmática, sin embargo si era muy lógico para nosotros la localización en retículo endoplásmico. Hubiese sido conveniente disponer de una trilocalización entre Vmp1, calnexina y zonula occludens 1 para poder dilucidar y entender mejor este resultado en mamíferos. Existe una salvedad a estos análisis que es el uso de células HEK293. Estas células poseen un citoplasma muy pequeño para estudiar una localización subcelular tan complicada como la de Vmp1, además el

hecho de tener que realizar el experimento con células en confluencia para observar claramente la localización en membrana de ZO-1 hace que el citoplasma de estas células disminuya aún más su tamaño y la dificultad para dilucidar la localización aumente.

Posteriores estudios comenzaron a establecer una clara relación de Vmp1 con autofagia (Ropolo et al. 2007). Mediante un anticuerpo anti-Vmp1 (diferente al usado en la localización mencionada anteriormente en membrana plasmática) y mediante la proteína de fusión Vmp1-GFP en células Hela, HEK293 y células acinares de páncreas de ratón, Vmp1 fue localizado en autofagosomas con una colocalización total con el marcador autofágico LC3II ("Microtubule-associated protein 1A/1B light chaín 3") y Beclin-1. Este dato se apoyaba en la coinmunoprecipitación de Vmp1 con Beclin-1 (Ropolo et al. 2007).

En clara oposición también a este resultado otro grupo abordó la localización de Vmp1 mediante la proteína de fusión Vmp1-GFP en células MEF (Itakura and Mizushima 2010). Mostraron una localización en estructuras autofágicas tempranas asociadas a retículo endoplasmico y zonas perinucleares similares al aparato de Golgi. Además en contraposición a la coinmunoprecipitación de Vmp1 con Beclin-1, este mismo grupo mostró mediante un gradiente de sacarosa que Vmp1 se encuentra en fracciones similares al marcador de retículo endoplásmico PDI y al de aparato de Golgi sintaxina 6 mientras que Beclin-1 se encuentra en fracciones diferentes (Itakura and Mizushima 2010). Sin embargo Beclin-1 es una proteína relacionada con múltiples procesos y por ello puede que no aparezca en fracciones autofágicas en suficiente cantidad como para ser detectado por esta técnica, además su presencia en estructuras autofágicas tempranas puede que sea dependiente de ayuno y el fraccionamiento no fuese realizado en estas condiciones (Itakura and Mizushima 2010).

Nuestro datos muestran como la proteína de rata Vmp1 complementa el fenotipo de la cepa mutante de *vmp1* en *Dictyostelium discoideum* con una clara colocalización en retículo endoplásmico con el marcador PDI. Esto nos indica que la proteína es funcional desde el retículo endoplásmico y que por lo tanto podría ser factible que estuviese localizada en el retículo endoplásmico de mamíferos (Calvo-Garrido et al. 2008b).

Es posible que Vmp1 en líneas celulares humanas se encuentre tanto en retículo endoplásmico como en autofagosomas conectando de alguna manera ambos orgánulos (posteriormente se abordará esta idea en profundidad).

¿Cuál es la función de Vmp1?

Vmp1 en *Dictyostelium discoideum*

Como anteriormente indiqué el estudio de una proteína de función desconocida es un largo camino donde cualquier tipo de abordaje bioinformático, el exhaustivo conocimiento de su localización, de sus posibles interactores o del funcionamiento de su cepa mutante son necesarios para plantear una hipótesis acerca de su posible función molecular. Ni en *Dictyostelium* ni en mamíferos se ha conseguido clarificar la función molecular de esta fascinante proteína, ya que en ambos modelos no se dispone de todos los datos o de datos verdaderamente esclarecedores.

Como se muestra en los capítulos 1, 2, 3 y 4, en *Dictyostelium discoideum*, Vmp1 es una proteína involucrada en muchísimos procesos cuya cepa mutante presenta defectos pleiotrópicos en multitud de aspectos del funcionamiento celular como la secreción proteica, la biogénesis de orgánulos como el retículo endoplásmico, el aparato de Golgi y la vacuola contráctil, el funcionamiento del sistema endocítico o la entrada en su fase de desarrollo. Además también encontramos múltiples defectos asociados al funcionamiento de la autofagia.

Con estos datos en la mano podemos pensar que Vmp1 es una proteína presente en el retículo endoplásmico que esta regulando múltiples procesos que se inician desde este orgánulo. También podríamos pensar que alguno de estos procesos que no discurren con normalidad es la causa fundamental de que el resto no funcionen correctamente, pero sin interactores y con un fenotipo extensamente pleiotrópico no podemos precisar más. Esta hipótesis es apoyada por los trabajos realizados en *Drosophila melanogaster*, donde TANGO5 (homólogo en *Drosophila melanogaster* a Vmp1) actuaría regulando la secreción proteica convencional y la organización del aparato de Golgi (Bard et al. 2006). No disponemos de datos preliminares en eucariotas superiores que puedan mostrar si por ejemplo la biogénesis del retículo

endoplásmico o del aparto de Golgi se encuentra afectada ya que solamente se ha abordado su relación con autofagia.

Vmp1 en autofagia

Inicialmente el grupo de M.I. Vaccaro describió a *vmp1* como un gen sobreexpresado en células acinares en la patología de pancreatitis aguda cuya sobreexpresión generaba en células cos7 vacuolización citoplásmica y muerte celular (Dusetti et al. 2002).

Posteriormente caracterizaron la posible relación de Vmp1 con autofagia en células Hela y células acinares de páncreas murino que expresaban constitutivamente Vmp1-GFP. Ambas líneas celulares mostraron, al igual que fue expuesto anteriormente en células cos7, que ésta sobreexpresión induce la formación de vacuolas citoplásmicas sin embargo ya no se menciona la inducción de muerte celular (Ropolo et al. 2007). Las membranas de estas vacuolas muestran la presencia de proteínas como LC3II, Beclin-1 y por supuesto Vmp1, con una colocalización completa de las tres. Concluyen que esta sobreexpresión de Vmp1-GFP en células Hela y acinares de páncreas deja de inducir muerte celular para pasar a activar autofagia (Ropolo et al. 2007). Este dato es apoyado con estudios de intereferencia mediante siRNA de Vmp1 en células Hela que impedía la activación de la autofagia en periodos de ayuno o por inducción mediante rapamicina.

Últimos avances realizados por este mismo grupo mostraron que esa regulación y activación de la autofagia por parte de la expresión constitutiva de Vmp1-GFP que tenía lugar en células acinares tenía un carácter protector (Grasso et al. 2010). La enfermedad de pancreatitis aguda se caracteriza por una activación temprana en células acinares de los zimógenos pancreáticos que viajan hacia el duodeno, la sobreexpresión mostrada inicialmente de *vmp1* en células acinares estaría dirigida a una activación de la autofagia con el fin de retirar del citoplasma estos zimógenos activados y proteger de ese modo a la célula. Este mecanismo protector autofágico en la enfermedad de pancreatitis aguda fue denominado zimofagia (Grasso et al. 2010).

Nuestros datos en relación a autofagia en *Dictyostelium* no se corresponden completamente con los expuestos anteriormente. En primer lugar la sobreexpresión de

Vmp1-GFP-TAP en *Dictyostelium* no muestra ningún fenotipo asociado, ni induce vacuolización, autofagia o muerte celular, sino que complementa el fenotipo de la cepa mutante de *vmp1* (Calvo-Garrido et al. 2008b, Calvo-Garrido, Carilla-Latorre and Escalante 2008a). Podríamos pensar que en *Dictyostelium* y mamíferos el funcionamiento de la proteína en relación a su sobreexpresión es diferente sin embargo la sobreexpresión de Vmp1-GFP en células MEF tampoco provoca muerte celular ni inducción de autofagia (Itakura and Mizushima 2010).

La cepa mutante de vmp1 en *Dictyostelium* muestra una gran acumulación del marcador autofágico GFP-Atg8 (Calvo-Garrido et al. 2008b), no una desaparición como en la interferencia mediante siRNA de *vmp1* en células Hela (Vaccaro et al. 2008). Además, experimentos previos realizados en nuestro laboratorio en células SH-SY5Y donde se interfirió la expresión de *vmp1*, muestran también acumulación de LC3II tanto en inmunocitoquímica como en western-blot. Los estudios realizados en células MEF intereferidas por siRNA para *vmp1* también han mostrado acumulación de LC3II (Itakura and Mizushima 2010).

La posible relación de Vmp1 como mecanismo protector en pancreatitis es consistente con los datos de sobreexpresión durante la enfermedad y la función de la autofagia como mecanismo protector que tiene capacidad de abarcar diferentes cargos citoplásmicos como proteínas mal plegadas, lípidos o patógenos. Sin embargo los datos aportados por este grupo contrastan fuertemente con los obtenidos en *Dictyostelium*, en nuestros abordajes previos en SH-SY5Y y los obtenidos por otro grupo en células MEF. De nuevo es posible que estas diferencias de deban a efectos dependientes de los diferentes tipos celulares.

Otros datos obtenidos en células MEF por el grupo de N.Mizushima donde la expresión de *vmp1* ha sido interferida mediante siRNA muestran un patrón de acumulación anormal de diferentes proteínas autofágicas como ULK1 (homólogo de la proteína Atg1 en mamíferos), WIPI-1 (homólogo de la proteína de levaduras Atg18a), DFCP1 y Atg16L1, pudiendo indicar un posible papel de Vmp1 en la maduración de estructuras autofágicas tempranas (Itakura and Mizushima 2010). Este dato podría tener mucho sentido en relación a nuestras observaciones de acumulación de la proteína de fusión GFP-Atg8 en *Dictyostelium*, ya que es posible que desde el retículo endoplásmico (localización apuntada por el grupo de Mizushima para Vmp1) se esté regulando la maduración de estructuras autofágicas.

EPG-3 fue identificado como la proteína homóloga a Vmp1 en *C.elegans*. Estudios de interferencia mediante siRNA sobre *epg-3* relacionaron a esta proteína con el control de la duración del omegasoma (estructura inicial en la formación del autofagosoma en la que interviene el retículo endoplásmico) (Tian et al. 2010). La ausencia de EPG-3 provocaba la acumulación de omegasomas algo que puede ser similar a la presencia de estructuras autofágicas aberrantes en células MEF, a nuestros datos previos en células SH-SY5Y y a la acumulación de GFP-Atg8 en *Dictyostelium discoideum*. Además estas células intereferidas mostraban en ayuno un aumento en los niveles de p62, algo que apoya nuestro hallazgo de agregados proteicos ubiquitinados positivos a p62 en *Dictyostelium* (Calvo-Garrido and Escalante 2010).

Complementación fenotípica en *Dictyostelium discoideum* y *C.elegans*

Como previamente habíamos descrito en *Dictyostelium discoideum* en relación a la complementación del fenotipo de la cepa mutante de *vmp1* por la proteína Vmp1 de rata, la cepa intereferida por siRNA de EPG-3 es complementada por la expresión de su homólogo humano.

La complementación del fenotipo mutante tanto en *Dictyostelium* como en *C.elegans* por la proteína homóloga de mamíferos pone de manifiesto la conservación funcional de la proteína Vmp1 a lo largo de la evolución. Inicialmente podríamos pensar que la menor complejidad celular de *Dictyostelium* o *C.elegans* podría mostrarnos de manera más clara y precisa la función de la proteína Vmp1, sin embargo son muchas las cuestiones por resolver en ambos modelos.

El entorno celular en el que se mueve Vmp1 en mamíferos es mucho más complejo, incluso una buena parte del conocimiento que tenemos de éste nos llega desde un modelo de fisiopatología como pancreatitis aguda, si a esto le sumamos además que la homología de la proteína no es completa, es complicado obtener una hipótesis funcional común a todos los modelos. Puede que la función esencial de Vmp1 siga presente en la proteína de mamíferos y por ello complemente el fenotipo mutante en *Dictyostelium* y *C.elegans* y que quizá también haya evolucionado para especializarse y adaptarse a nuevos procesos relacionados con cáncer o pancreatitis aguda.

Claramente se abre aquí una pregunta, ¿complementaría la proteína Vmp1 de *Dictyostelium discoideum* o EPG-3 de *C.elegans* el fenotipo interferido de *vmp1* en células de mamífero? Quizá la respuesta a esta pregunta podría arrojar luz acerca de si Vmp1 mantiene su función inicial o evolutivamente ha existido un cambio importante.

¿Está Vmp1 involucrada en otras funciones además de autofagia?

La variedad de fenotipos observados en los modelos donde *vmp1* ha sido interferido o eliminado abren la interesante reflexión sobre si Vmp1 tiene otras funciones más alla de la autofagia.

Solamente en *Dictyostelium discoideum* y en *Drosophila melanogaster* se han mostrado datos de una posible implicación de Vmp1 en procesos diferentes a autofagia. El fenotipo mostrado por la cepa K.O. de *vmp1* en *Dictyostelium* (es el único modelo que muestra una eliminación total de la expresión de *vmp1*) y la interferencia por siRNA en *Drosophila melanogaster* indican una posible disfunción general de tráfico de membranas con origen en el retículo endoplásmico (Bard et al. 2006).

La cepa mutante de *vmp1* muestra fenotipos similares a otros mutantes autofágicos como *atg5*, atg6, atg7, *atg8* o *atg1* (presencia de agregados proteicos, etapa de desarrollo aberrante o ausente, crecimiento más lento o una supervivencia en ayuno reducida) (Calvo-Garrido and Escalante 2010, Otto et al. 2004). Sin embargo el fenotipo de la cepa mutante de *vmp1* o de *atg1* muestran fenotipos mucho más severos. La proteína Atg1 ha sido relacionada con otros procesos diferentes a autofagia además de ser una de las proteínas que parece actúan como unión entre autofagia y el estado metabólico de la célula (Egan et al. 2010), por lo que podría ocurrir algo similar con Vmp1, que su función vaya más allá de la autofagia. De todos modos la mayor severidad del fenotipo de las cepas mutantes de *vmp1* y *atg1* puede deberse también a que actúen en una etapa más inicial de la ruta autofágica, algo que indican experimentos realizados en células MEF con Vmp1 y ULK1 (Itakura and Mizushima 2010).

Sería interesante comprobar si algunos de los procesos aberrantes no relacionados con autofagia de la cepa mutante de *vmp1* (como la biogénesis de orgánulos o la secreción proteica) aparecen en los mutantes autofágicos *atg1*, *atg5*, *atg6*, *atg7* y *atg8* y con que severidad. Y por supuesto si también aparecen en la interferencia de *vmp1* en células de mamífero.

Otras implicaciones patológicas de Vmp1

Cáncer

Como ya se indicó en la introducción *vmp1* ha sido encontrado desregulado en diferentes procesos cancerígenos (Higgins et al. 2003). Sin embargo solamente el trabajo que localizó a Vmp1 en membrana plasmática abordó de un modo más profundo su relación con aspectos relacionados con cáncer como invasividad y metátasis (Sauermann et al. 2008). La expresión de *vmp1* disminuye en tumores metastáticos respecto a tumores primarios, además, estudios de interferencia por siRNA de *vmp1* en células no invasivas de riñón (Caki-2) mostraron una importante pérdida de adhesión celular y un aumento de la capacidad invasiva de las células y consecuentemente un aumento en su potencial metastático (Sauermann et al. 2008).

Teniendo en cuenta estos datos y la sorprendente localización en membrana plasmática de Vmp1 en células HEK293, es factible que exista una relación directa de Vmp1 con invasividad tumoral y de ese modo con metastásis. Actualmente se ha relacionado autofagia con cáncer y con la degradación selectiva de membrana plasmática por autofagia, sin embargo si hubiese una activación específica de autofagia para disminuir la adhesión celular y promover la invasividad y migración celular esperaríamos un aumento de la expresión de *vmp1*, no una disminución. Otra opción es que se trate de una función específica de Vmp1 en membrana plasmática en algunos tipos celulares cancerígenos. También puede ocurrir que la membrana plasmática, al igual que el retículo endoplásmico, actúe como donador de membranas para la formación de autofagosomas (Tooze and Yoshimori 2010).

La colocalización y coinmunoprecipitación de Vmp1 con ZO-1 fue realizada en células HEK293, sin embargo el estudio del funcionamiento de la invasividad tumoral y

metástasis se realizó en células Caki-2, hubiese sido más adecuado disponer de los datos en ambos tipos celulares.

Estrés celular

El estrés celular desencadena diferentes tipos de respuestas por parte de la célula incluída la autofagia. Diferentes estudios de este tipo han mostrado un aumento de la expresión de *vmp1*: estudios de isquemia inducida en riñón de modelos murinos (Dusetti et al. 2002), de células de cáncer de mama tratadas con el anti-inflamatorio indometazina (Glunde, Jie and Bhujwalla 2006) (*vmp1* es el segundo gen más activado en el tratamiento con indometazina) y de células PC12 tratadas con MPP+ (tóxico que actúa sobre el complejo I mitocondrial y genera daños celulares parecidos a la enfermedad de Parkinson) (Xu et al. 2005).

Estos datos sugieren que quizá *vmp1* aumente su nivel de expresión rápidamente ante algunos fenómenos de estrés celular. Autofagia y estrés celular son dos aspectos ampliamente relacionados sin embargo en los estudios por microarryas con indometazina y MPP+ donde se aporta una amplia tabla de genes desregulados no aparece ninguno aparte de *vmp1* de los más típicamente relacionados con autofagia. Sería interesante inducir diferentes tipos de estrés celular y comparar los niveles de proteínas como “heat shock proteins” en células “wild type” y mutantes o interferidas para *vmp1*. De ese modo podríamos ver si Vmp1 está modulando o está involucrado en la respuesta ante un estrés o daño celular.

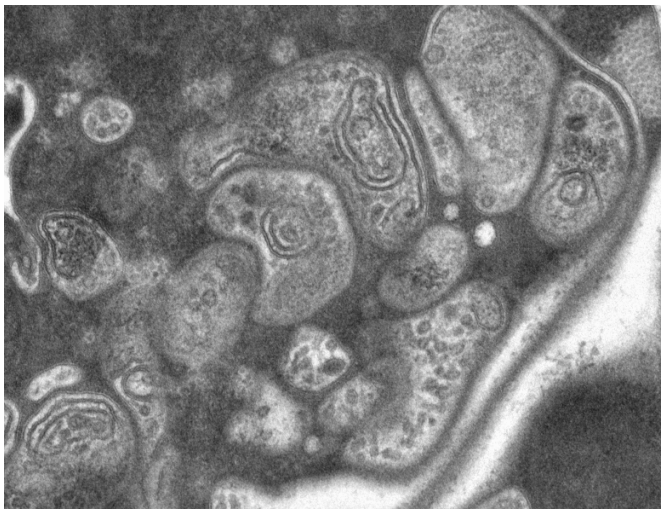
Vmp1 es una proteína fascinante y tremendamente compleja, los abordajes realizados para entender su función han deparado resultados extremadamente interesantes que invitan a seguir trabajando en la búsqueda de su función y su posible papel en diversas patologías, sin embargo pese a ser interesantes no son suficientes para establecer una clara hipótesis funcional para Vmp1 (tabla 1).

Es muy posible que Vmp1 en organismos como *Drosophila melanogaster*, *C.elegans* o *Dictyostelium discoideum* regule, desde el retículo endoplásmico, la formación de todo tipo de vesículas que tengan origen en éste, sin embargo al subir en la escala evolutiva puede que su función haya sufrido un proceso de especialización relacionado con la autofagia y su localización haya cambiado a estructuras reticulares

específicas de interacción directa con la formación y maduración de los autofagosomas.

Tipo celular	Localización	Interactores	Función	Sobreexpresión de *Vmp1*	Referencias
Cos7	ER,Golgi, vacuolas citoplásmicas	No descritos	Factor pro-apoptótico	Vacuolización y muerte celular	(Dusetti et al. 2002)
Hela	Autofagosomas	Beclin-1	Autofagia	Vacuolización e inducción de autofagia	(Ropolo et al. 2007)
C.Acinares	Autofagosomas	USPX9	Autofagia y pancreatitis aguda	Inducción de autofagia y protección frente a p.aguda	(Grasso et al. 2010)
MEF	Zonas de formación autofagosomas en ER	No Beclín1	Autofagia	Ningún efecto	(Itakura and Mizushima 2010)
D.*melanogaster*	No analizado	No descritos	Secreción proteíca. Organización del aparato de Golgi.	No analizado	(Bard et al. 2006)
C.elegans	No analizado	No descritos	Autofagia, duración omegasoma	Complementación cepa mutante *epg*-3.	(Tian et al. 2010)
HEK293T	RE y membrana plasmática	ZO-1	Adhesión celular e invasividad tumoral	Vacuolización y muerte celular	(Sauermann et al. 2008)
D.*discoideum*	RE y autofagosomas	No descritos	Fenotipo pleiotrópico	Complementación mutante *vmp1*.	(Calvo-Garrido and Escalante 2010)

Tabla 1. Localizaciones, interactores, posibles funciones y efecto de la sobreexpresión de Vmp1 en las líneas celulares y modelos donde ha sido estudiado.



CONCLUSIONES

CONCLUSIONES

1. La proteína Vmp1 de *Dictyostelium discoideum* es una proteína transmembrana localizada en el retículo endoplásmico y que colocaliza con una fracción de los autofagosomas.

2. La cepa deficiente de *vmp1* en *Dictyostelium discoideum* muestra defectos fenotípicos en la biogénesis de orgánulos como la vacuola contráctil, la estructura del retículo endoplásmico y el aparato de Golgi, así como en el funcionamiento de la macropinocitosis, la fagocitosis, la secreción proteica convencional y la fase de desarrollo multicelular.

3. La supervivencia en ayuno y la formación de células tallo *in vitro* son dos procesos dependientes de autofagia. Ambos procesos son deficientes en la cepa mutante de *vmp1*.

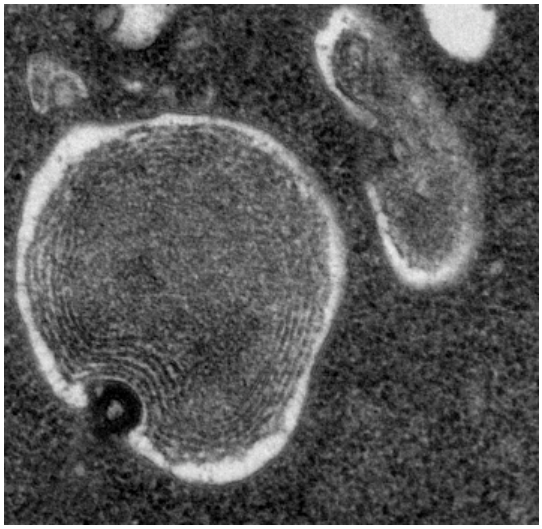
4. La expresión de la proteína de fusión Vmp1-GFP-TAP complementa la cepa deficiente de *vmp1* en *Dictyostelium discoideum*, del mismo modo que lo consigue la proteína Vmp1-GFP-TAP de rata. Este hecho implica una conservación funcional de Vmp1 entre *Dictyostelium* y mamíferos.

5. El patrón del marcador autofágico GFP-Atg8 en la cepa “wild type” presenta mayoritariamente un aspecto punteado en presencia de alimento mientras que en ayuno prevalece la presencia de estructuras vesiculares más desarrolladas.

6. El patrón del marcador autofágico GFP-Atg8 en la cepa mutante de *vmp1* es aberrante, encontrándose en agregados. Estos agregados colocalizan con proteínas ubiquitinadas donde está presente la proteína homóloga de humanos p62, un marcador de disfunción autofágica.

7. Existe una correlación positiva entre el tamaño de los agregados ubiquitinados presentes en mutantes autofágicos como *vmp1*, *atg1*, atg5, atg6, *atg7* y *atg8* y la severidad de su fenotipo. Las cepas mutantes de *vmp1* y *atg1* presentan fenotipos más severos y mayores agregados, las de *atg5* y *atg7* fenotipos menos severos y agregados más pequeños y las de *atg6* y *atg8* fenotipos aún menos severos y ausencia de agregados.

8. La alta homología existente entre muchas de las proteínas humanas relacionadas con autofagia y sus homólogos en *Dictyostelium discoideum* y las nuevas técnicas y conocimientos aportados durante esta tesis doctoral hacen de *Dictyostelium discoideum* un modelo válido y necesario para el avance en el conocimiento de la autofagia en eucariotas superiores y su relación con procesos fisiopatológicos.



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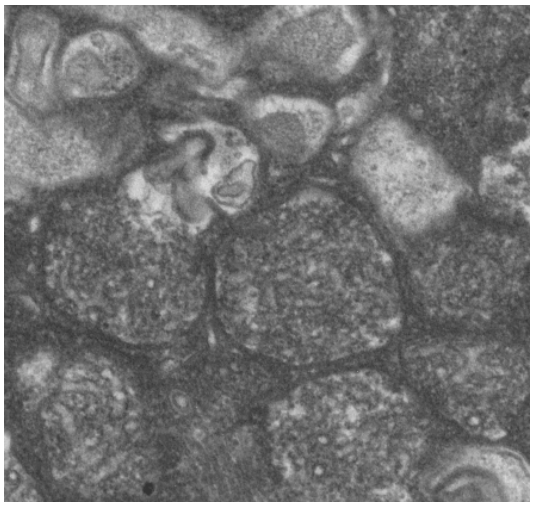

ANEXO: ARTÍCULOS PUBLICADOS II

CAPÍTULO 5: *DICTYOSTELIUM* TRANSCRIPTIONAL RESPONSES TO *PSEUDOMONAS AERUGINOSA*: COMMON AND SPECIFIC EFFECTS FROM PAO1 AND PA14 STRAINS

Pseudomonas aeruginosa es una de las bacterias patógenas oportunistas más importantes desde el punto de vista sanitario ya que muchas de las infecciones postoperatorias se deben a ella. Existen dos cepas PAO1 y PA14 que han sido usadas como modelos de virulencia, siendo PA14 más virulenta que PAO1.
Estas diferencias parecen atribuibles a una serie de variaciones en el genoma de estas dos cepas sin embargo poco o nada se sabe de cómo afectan al mecanismo de defensa del huésped.

Células de *Dictyostelium discoideum* fueron incubadas en presencia y ausencia de ambas cepas por separado durante 4 horas, estudiándose por micro-arrays la respuesta de *Dictyostelium discoideum* a estos patógenos.

Se obtuvieron una serie de genes que aumentaban o disminuían su expresión de manera independiente al uso de PA14 o PAO1 mientras que otros fueron específicos del uso de una u otra cepa. Un amplio porcentaje de ellos formaba parte de genes relacionados con metabolismo.

Este estudio de micro-arrays fue validado por PCR cuantitativa de 7 de los genes desregulados.

BMC Microbiology

Research article

Dictyostelium transcriptional responses to *Pseudomonas aeruginosa*: common and specific effects from PAO1 and PA14 strains

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most relevant human opportunistic bacterial pathogens. Two strains (PAO1 and PA14) have been mainly used as models for studying virulence of *P. aeruginosa*. The strain PA14 is more virulent than PAO1 in a wide range of hosts including insects, nematodes and plants. Whereas some of the differences might be attributable to concerted action of determinants encoded in pathogenicity islands present in the genome of PA14, a global analysis of the differential host responses to these *P. aeruginosa* strains has not been addressed. Little is known about the host response to infection with *P. aeruginosa* and whether or not the global host transcription is being affected as a defense mechanism or altered in the benefit of the pathogen. Since the social amoeba *Dictyostelium discoideum* is a suitable host to study virulence of *P. aeruginosa* and other pathogens, we used available genomic tools in this model system to study the transcriptional host response to *P. aeruginosa* infection.

Results: We have compared the virulence of the *P. aeruginosa* PAO1 and PA14 using *D. discoideum* and studied the transcriptional response of the amoeba upon infection. Our results showed that PA14 is more virulent in *Dictyostelium* than PA01using different plating assays. For studying the differential response of the host to infection by these model strains, *D. discoideum* cells were exposed to either *P. aeruginosa* PAO1 or *P. aeruginosa* PA14 (mixed with an excess of the non-pathogenic bacterium *Klebsiella aerogenes* as food supply) and after 4 hours, cellular RNA extracted. A three-way comparison was made using whole-genome *D. discoideum* microarrays between RNA samples from cells treated with the two different strains and control cells exposed only to *K. aerogenes*. The transcriptomic analyses have shown the existence of common and specific responses to infection. The expression of 364 genes changed in a similar way upon infection with one or another strain, whereas 169 genes were differentially regulated depending on whether the infecting strain was either *P. aeruginosa* PAO1 or PA14. Effects on metabolism, signalling, stress response and cell cycle can be inferred from the genes affected.

Conclusion: Our results show that pathogenic *Pseudomonas* strains invoke both a common transcriptional response from *Dictyostelium* and a strain specific one, indicating that the infective process of bacterial pathogens can be strain-specific and is more complex than previously thought.

Background

Nosocomial infections caused by opportunistic pathogens are one of the most important health problems in developed countries. Depending on the geographic location, *P. aeruginosa* is the first or second causative agent of nosocomial infections [1,2]. *P. aeruginosa* infects patients suffering from AIDS, people at intensive care units, and burned people among others, and is the major cause of morbidity and mortality in patients with cystic fibrosis, the most prevalent hereditary disease in Caucasian populations [3]. A successful infection by this type of pathogens depends on the interplay of multiple factors including the susceptibility of the host, the virulence of the strain and its resistance to antibiotics [4]. Previous work has shown that the physiological fitness and the virulence of *P. aeruginosa* and other opportunists are affected by the expression of antibiotic resistance mechanisms such as MDR-pumping systems [5-8].

The pathogenicity of *Pseudomonas aeruginosa* involves various components operating at different levels. The flagella and *pili* facilitate contact with the bacterium's cell target and play a role in its adhesion, which is a critical step in the infection [9,10]. After contact, the type III secretion system is able to inject into the cytoplasm of the target cell a series of cytotoxic molecules that act at various levels. The mechanism of action involves, in many cases, the presence of host cofactors still unidentified [11]. Other virulence factors involve products secreted into the extracellular medium by systems I and II such as elastase, alkaline phosphatase and exotoxin A among others. The expression of many of these virulence factors is regulated by a mechanism of bacteria-to-bacteria cell signalling known as quorum-sensing [12]. Despite the functional and genomic similarity among different *P. aeruginosa* strains [13,14], some differences in their pathogenicity have been observed [15]. For example, the clinical isolate PA14 is more virulent than PAO1 in a wide range of hosts [15-17]. It has been shown that the genome of PA14 contains two pathogenicity islands that are not present in PAO1 and it has been proposed that the virulence in this organism (and the difference between PA14 and PAO1) is the result of a pool of pathogenicity genes interacting in various combinations in different genetic backgrounds [15]. In spite of these suggestions, the cause of the different virulence behavior of PAO1 and PA14 is not yet fully understood.

Although most of the work on pathogenesis has been focused on understanding the bacterial factors that render a virulence phenotype, increasing attention is being paid to the host and those aspects connected to the susceptibility or resistance to infection. Understanding the host-pathogen relationship, at both the cellular and molecular level, is essential to identify new targets and develop new strategies to fight infection. Molecular analysis of host-pathogen interactions would benefit from the use of model systems allowing a systematic study of the factors involved. In this regard the social amoeba *D. discoideum* has proven particularly useful for its ease of handling, genetic tractability [18-22] and fully sequenced genome [23].

D. discoideum is a soil microorganism that feeds on bacteria by phagocytosis. The interaction between bacteria and their natural predators (*Dictyostelium*, other protists and worms) is believed to have shaped both predators [24] and bacterial evolution. As a consequence, some of the mechanisms developed by bacteria to avoid the activity of their natural predators in the environment might have been adapted later in evolution to allow the infection of higher organisms such as humans [25]. Specifically, it was found that the quorum-sensing mechanisms and type III secretion, which are essential factors in the infectivity to humans are also responsible for the infectivity of *P. aeruginosa* in *D. discoideum* [18,20,21].

Our previous studies have shown the utility of this model system of infection to analyze the virulence of other opportunistic pathogens like *Stenotrophomonas maltophilia* [7]. It has been also demonstrated the validity of *D. discoideum* as a model of infection by intracellular pathogens such as *Legionella, Cryptococcus and Mycobacterium* [19,22]. Consequently, the conservation of the mechanisms of infection needed to infect mammals and *D. discoideum* in a wide variety of pathogens reinforces the use of this system as a valid model to study host-pathogen relations. We have used whole-genome *D. discoideum* microarrays to study global host transcription upon infection with *Pseudomonas aeruginosa* PAO1 and PA14 to determine whether or not transcription is being affected as a defense mechanism or altered in the benefit of the pathogen.

Results

Pseudomonas aeruginosa *strains PAO1 and PA14 show a different virulence behavior in* D. discoideum

PAO1 and PA14 are two clinical isolates of *P. aeruginosa* frequently used as model strains to analyze the virulence of this bacterial pathogen. Since they behave differently in some aspects dealing with the expression of virulence determinants, we wanted to compare the differential response of the host to these strains. For this purpose, we made use of *D. discoideum* as a model for virulence. As a first step a plating assay of virulence was set up. Figure 1 shows a representative experiment of three independent assays in which *D. discoideum* cells were grown in association with bacteria on nutrient SM plates. *Klebsiella aerogenes*, a non -pathogenic bacteria, was used as an appropriate food supply and *P. aeruginosa* mixed at the indicated proportions. An effect in the size of the clearing

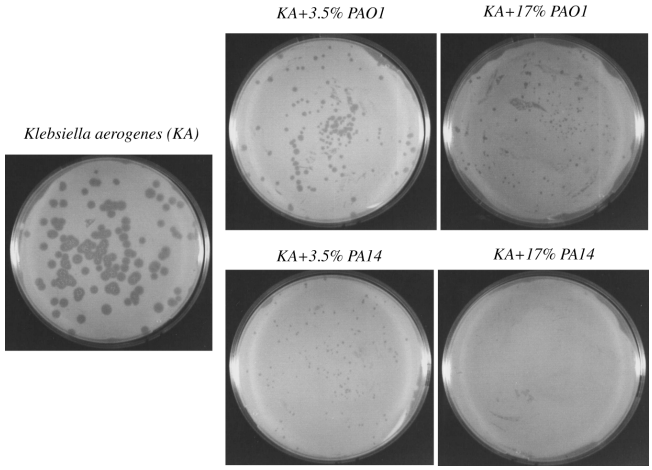

Figure 1
PA14 is more virulent than PAO1 in SM-plating assay. Approximately 100 *D. discoideum* cells were cultivated in SM-plates with the indicated proportion of *Klebsiella* and *Pseudomonas* strains (PAO1 or PA14) previously grown and adjusted to the same optical density. Plates were maintained at 22°C for 5 days. Growth of *D. discoideum* is severely affected by the presence of *Pseudomonas* but the inhibition is stronger when PA14 is used.

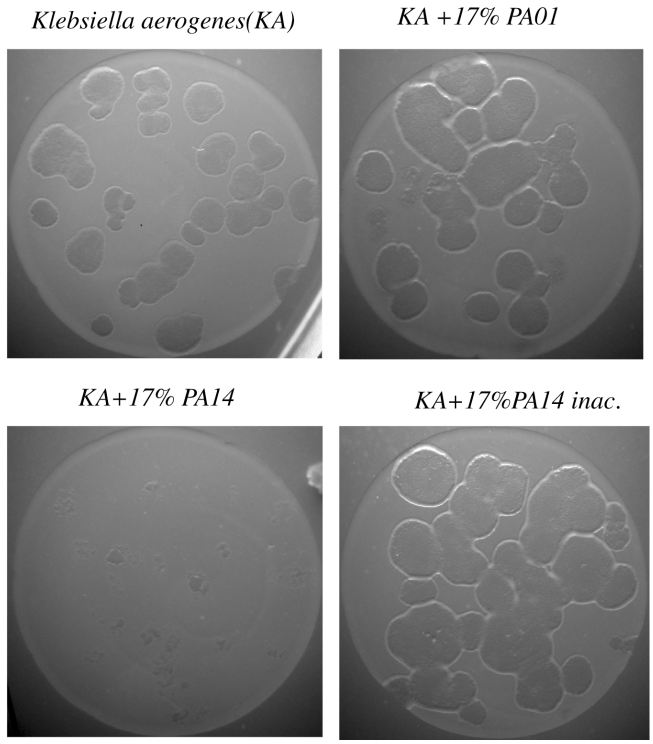

Figure 2
PA14 is more virulent than PAO1 in PDF-agar plating assay. *D. discoideum* cells were cultivated in non-nutrient agar on a lawn of *Klebsiella* and *Pseudomonas* (PAO1 and PA14) at the indicated proportion. Under these conditions PA14 maintain a high virulence as seen by the strong inhibition of *D. discoideum* growth. Heat inactivated PA14 is used as a control.

plaques could already be seen when only 3.5% of *P. aeruginosa* cells were mixed with 96.5% of *K. aerogenes* cells and this effect was even clearer using 17% of *P. aeruginosa* cells. When the behavior of the strains was analyzed in more detail, it was found that PAO1 is reproducibly more permissive than PA14 as observed by the higher growth of *D. discoideum* on PAO1. The differences in the area of the cleared bacterial lawn between PAO1 and PA14 were measured for the condition corresponding to the 3.5 % mixture. The average area and the standard deviation were 1.65 ± 1.2 mm^2 for PAO1 and 0.11 ± 0.07 mm^2 for PA14 (the number of clear plaques measured in each condition was 50). The significance of differences between groups as determined by Student's *t*-test was $p < 10^{-8}$. To further confirm these results a different plating assay was performed on non-nutrient agar. PAO1, PA14 and *K. aerogenes* were previously grown in LB overnight, washed out of the media by centrifugation and deposited with *D. discoideum* cells in agar plates at the indicated proportions. Under these conditions the difference in the virulence between PAO1 and PA14 was even more evident as shown in a representative experiment in Figure 2. Interestingly PAO1 is permissive to *D. discoideum* growth under these non-nutrient conditions. However, PA14 still shows a strong virulence against *D. discoideum*. All together these results suggest that PA14 is more virulent than PAO1 in the *D. discoideum* model of virulence.

Pseudomonas aeruginosa *induces a specific gene expression response in* Dictyostelium

Little is known about the interplay between the host and the pathogen in terms of gene expression responses. We wanted to determine if there is a specific gene expression response of *D. discoideum* to their interaction with *P. aeruginosa*. *D. discoideum* cells were exposed to *P. aeruginosa* strains PAO1 and PA14 mixed with an excess of *K. aerogenes* in HL5 for 4 hours. *K. aerogenes* alone was used as a control to which the gene expression levels were compared. RNA was extracted from *D. discoideum* and used to study the global pattern of gene expression using whole-genome *D. discoideum* microarrays (see Additional file 1 for the complete data). Using a $P < 0.05$ cutoff, there were 752 genes whose expression was significantly different between the PAO1-treated cells and the controls and 624 genes between PA14-treated cells and controls (Table 1 summarizes the results at different P values and log-ratios). The heat map shown in Figure 3 indicates that the responses were broadly comparable between the two strains with very few genes oppositely altered in the two

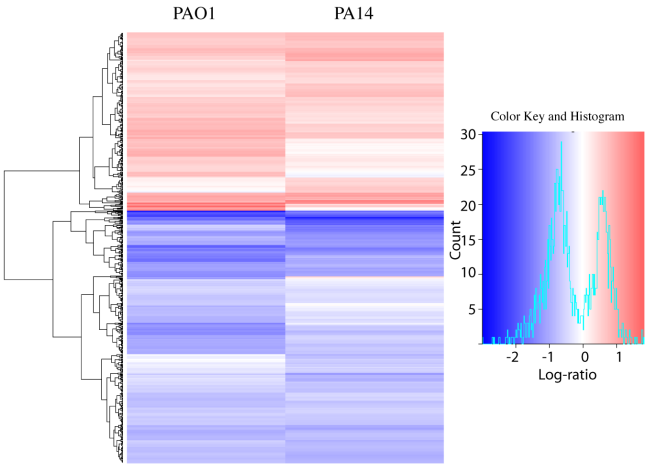

Figure 3
Pseudomonas aeruginosa* induces gene expression changes in *Dictyostelium. Heat map comparing the genes significantly altered ($p < 0.05$) between PAO1-treated cells versus control (975) and PA14-treated cells versus control (838). Each row of the plot is a gene and was colored according to the log2ratio of expression with red meaning up-regulation in relation to the controls and blue downregulation. The histogram shows the range of changes in a log2 scale. The data presented are for the three independent experiments combined. The heatmap was generated using the heatmap.2 function of the gplots package in R [47]. The dendrogram was generated using Euclidean distance and the "complete" agglomeration method.

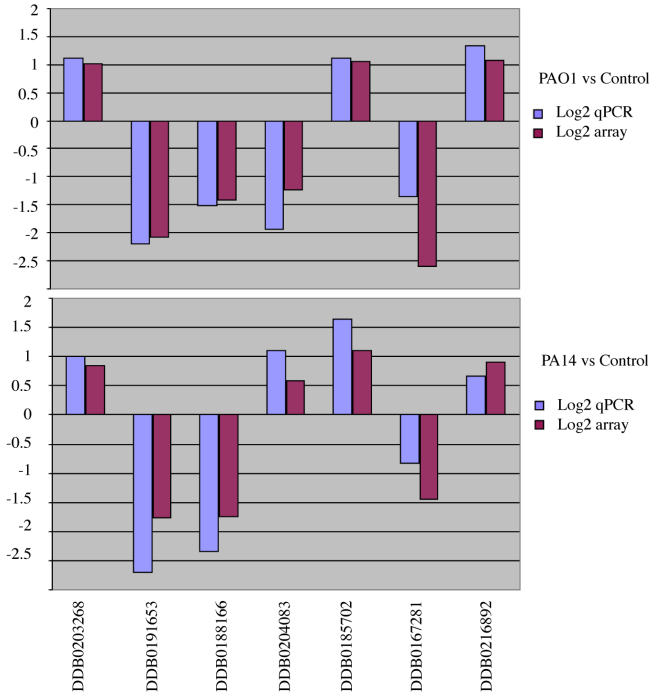

Figure 4
Correlation of microaray and real-time PCR. Real-time PCR measurements of the mRNA levels for seven representative genes whose expression were affected in the array. Upper panel shows a direct comparison of the changes in a log2 scale for PAO1 versus control and the lower panel shows the same genes for PA14 versus control. Blue bars corresponded to quantitative real time PCR and the purple bars to the array data. The array data and the real time PCR displayed are the combination of three independent biological experiments. The correlation coefficients were: $R^2 = 0.87$ for PAO1 and $R^2 = 0.91$ for PA14.

conditions. The differences in the gene expression are approximately in the range between four-fold repression and three-fold induction (log-ratios between of -2 to +1.5 as shown in the histogram of Figure 3). These results were validated by real time PCR of the same samples used for the transcriptomic assays, measuring the expression of 7 representative genes that were up-regulated or down-regulated in the different conditions. Figure 4 shows a good correlation between the data obtained from the microarray transcriptomic experiment as compared with that obtained by quantitative RT-PCR. Although the log-ratio changes in the gene expression showed some differences the overall trend were consistent, supporting the reliability of our data.

Common and specific responses of D. discoideum *to the infection with PAO1 and PA14 strains*

As shown in Table 1 there were 364 genes that showed similar differential regulation with both bacterial strains compared with the controls (labeled as PAO1+PA14 vs control). We have considered in the analysis those genes showing differences in log-ratios that are higher than +0.5 or lower than -0.5. Interestingly the expression of another group of 169 genes (labeled as PAO1 vs PA14) was different depending on whether the infecting strain was PAO1 or PA14. We have studied in detail both groups by manual annotation and categorizing using the extended categorization for *D. discoideum* previously described [26]. Genes of unknown function and those showing weak homologies were not included in the list. Table 2 contains the genes that were similarly regulated upon infection with any of both strains, and in Figure 5 the genes are categorized by function (see Additional file 2 for the complete data). The first interesting conclusion from this experiment is the existence of a common transcriptional response that affects many different genes that are involved in a wide range of functions. The proportion of the genes that were downregulated by the treatment with both strains of *P. aeruginosa* is higher (258 genes) compared to those upregulated (106 genes). This difference is more evident in categories such as stress response and transport (Figure 5).

Table 1: Differential genes at p < 0.05 and different Log 2 ratios

	PAO1 vs Control	PA14 vs Control	PAO1+PA14 vs Control	PAO1 vs PA14
Log 2 ratio	**752**	**623**	**364 (Table 2)**	**169 (Table 3)**
(>+0.5 or <-0.5)	461 down 291 up	396 down 227 up	258 down 106 up	60 down 109 up
Log 2 ratio	**150**	**125**	**70**	**35**
(>+1 or <-1)	126 down 24 up	105 down 20 up	66 down 4 up	14 down 21 up

Table 3 displays the D. discoideum genes whose expression changed differentially between PAO1 and PA14 infection and Figure 6 shows the number of genes in each category (see Additional file 3 for complete data). In general a higher proportion of the genes showed a higher level of expression by PAO1 infection (109 genes) when compared with the levels observed by PA14. On the other hand, 60 genes behaved oppositely showing lower levels of expression upon exposure to PAO1 compared to those levels obtained after PA14 infection. Interestingly, all the genes represented in the categories stress-response and protein targeting had a higher level of expression in the cells exposed to PAO1 compared to PA14. The behavior of these genes in comparison with the control is also displayed in Table 3.

Discussion

P. aeruginosa is able to infect *D. discoideum* cells using several virulence traits that are similar to those used to infect mammalian cells and other hosts [18]. The clinical *P. aeruginosa* isolates PA14 and PAO1 have been used independently to study the infection of *Dictyostelium* by *Pseudomonas* in two different laboratories [20,21]. However, no direct comparison had been reported so far between these strains in this pathogenicity model. We now report that PA14 is indeed more virulent in *D. discoideum* using different plating assays. Since *P. aeruginosa* is phagocytosed at much lower rate than the non-pathogenic *K. aerogenes* (commonly used to grow *Dictyostelium*) [20], the assays were designed to provide sufficient food to *D. discoideum* to avoid cell starvation. Thus, *K. aerogenes* was always used in excess together with the pathogenic strains. In the first assay (Figure 1) a nutrient plate was used to allow the growth of bacteria and *D. discoideum* simultaneously. Under these conditions the presence of PA14 inhibits *D. discoideum* growth to a greater extent than PAO1. To avoid differences in the growth rates between bacteria that might alter their final proportions, a non-nutrient assay was performed (Figure 2). In these experiments, *D. discoideum* feed on bacteria that have been previously grown and deposited at different proportions in non-nutrient agar. Interestingly, PAO1 is not virulent in this condition suggesting that bacterial growth is necessary for the expression of the virulence in this strain. However, even in these conditions PA14 is capable of inhibiting *D. discoideum* growth. Some studies have suggested that PA14 pathogenicity is multifactorial and required the action of multiple virulence mechanisms [15,27]. These differences between strains prompted us to study the transcriptional profile of *D. discoideum* upon infection with PAO1 and PA14 to gain insights not only into the possible common transcriptional response but also into any specific response that could explain the observed differences in their virulence. Pilot experiments showed that 4 hours of exposure of *D. discoideum* cells to either *Pseudomonas aeruginosa* strains did not result in any apparent cell death or change in cell morphology (data not shown). Since we wanted to study the early transcriptional response we chose this short time of exposure to avoid changes due to cell death. The existence of a rapid gene expression response between 1–6 hours upon exposure of *D. discoideum* cells to *Legionella*, an intracellular pathogen, has also been described [28].

Our results show the existence of a common transcriptional response to the infection with *P. aeruginoa* PAO1 or PA14 that affects 364 genes grouped in many different cellular functions. The complexity of the observed transcriptional changes could be the result of the induction of *D. discoideum* defensive responses or triggered by *P. aeruginosa* to make a less hostile cell environment that would support a better survival of the pathogen. In this scenario downregulation of genes involved in stress response might be beneficial for a successful infection. Interestingly, we have observed a clear down-regulation of genes dedicated to stress in the common response to PAO1 and PA14 but also in the specific response to the more virulent strain PA14. For example the gene coding for Strictosidine synthase (DDB0185428) which is involved in the synthesis of alkaloids related to defense mechanisms in plants [29], Trap1 (DDB0169033) that plays a central role in cell cycle regulation and differentiation [30] or the genes coding for lysozymes involved in bacterial degradation (DDB0167491) [31], to mention just a few.

Besides stress response other categories are affected by an overall downregulation such as metabolism, translation and transport facilitation. A subcategorization of the genes included in metabolism (see supplementary table 2) showed that all the genes coding for proteins involved in nucleotide metabolism were downregulated in the common response suggesting an effect on cell prolifera-

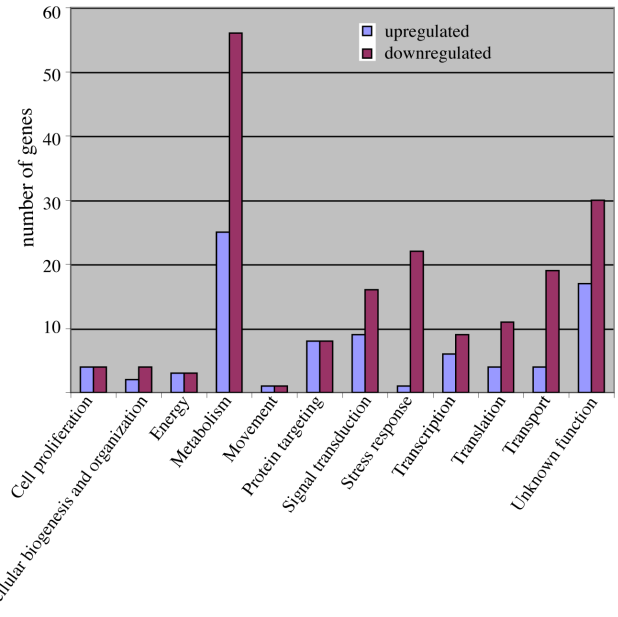

Figure 5
Functional categories of the genes affected similarly by the exposure with PAO1 and PA14. The genes whose expression was altered by PAO1 and PA14 were manually annotated (see Table 2) and grouped in functional categories. The size of the blue bars indicates the number of genes upregulated in each category related to the control and purple bars the number of genes downregulated.

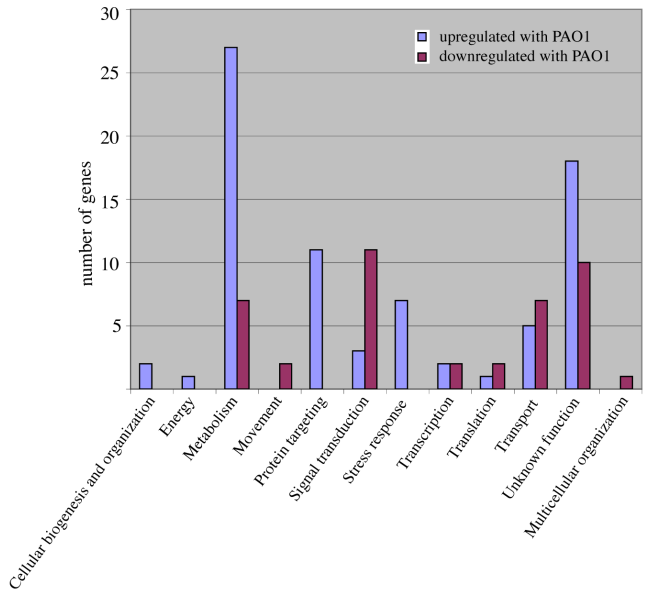

Figure 6
Functional categories of the genes differentially affected by the exposure to PAO1 and PA14. The genes whose expression was differentially altered in PAO1 versus PA14 were manually annotated (see Table 3) and grouped in functional categories. The size of the blue bars indicates the number of genes upregulated in PAO1 versus PA14 in each category and purple bars the number of genes downregulated in PAO1 versus PA14.

tion. Moreover, a number of other downregulated genes are directly involved in cell growth as demonstrated functionally in previous studies. This is the case for example of DDB0192001 (ppkA, polyphosphate kinase), whose disruption leads to reduced growth on bacteria [32,33], DDB0186120 (gcsA, glutamylcysteine synthetase), which is essential for cell growth as mutants in the gene are not viable in the absence of glutathione [34]. DDB0168860 (sgkA, sphingosine kinase) that is involved in cell proliferation [35], among others that have been annotated in Supplementary Table 2, 3.

Two different expression microarray analysis in mammals upon infection with *Pseudomonas aeruginosa* have been reported. In the first report epithelial cells were exposed to the pathogen for 3 hours, a short exposure similar to our experimental design. Unfortunately the number of genes represented in the array was very limited (1500 cDNAs) [36]. Only 22 genes were differentially regulated and we have not found any homologous gene in common. The other work reported the analysis of *Pseudomonas aeruginosa* corneal infection using an oligonucleotide microarray [37]. This experiment is not directly comparable to ours since a long exposure to the pathogen (1 day) was performed to assure an infection process. As a consequence most of the regulated genes were associated with the immune response and apoptosis, aspects that are not present in *Dictyostelium*.

D. discoideum is also susceptible to the infection by *Legionella pneumophila*, a facultative intracellular parasite, which uses different infective mechanisms from *P. aeruginosa*. It is important to note here that the transcriptional response of *D. discoideum* upon infection with *Legionella* [28] was essentially different to the one we report for *P. aeruginosa*. Only 8 genes were found to be altered in both experiments (DDB0186332, DDB0219578, DDB0167879, DDB0205386, DDB0185740, DDB0167345, DDB0201617, DDB0202615). This indicates that the host response is rather specific of the type of infection and the bacterial pathogen involved. Nevertheless, some responses can be also common. For instance, DDB0202615 (nramp1, natural resistance-associated macrophage protein) whose expression is downregulated in PAO1 and PA14, plays an important role in Legionella infection since the null mutant has increased sensitivity to the infection [38]. Nramp1 transports metal cations out of the phagolysosome in an ATP-dependent process. This

Table 2: Genes differentially expressed upon infection with PAO1 and PA14 versus *Klebsiella*

Gene ID	Gene function and name	Log 2 ratio: PAO1-C	PA14-C
Cell proliferation			
DDB0216882	Cyclin-dependent kinase regulatory	-0,89	-0,97
DDB0188449	cdc40, conserved splicing factor	0,68	0,78
DDB0205486	CDK family protein kinase	0,81	0,89
DDB0168249	cdk1, "cyclin-dependent_kinase, p34-cdc2_protein"	-0,78	-0,83
DDB0216532	cdk10;"putative_CDK_family_protein_kinase	0,91	1,08
DDB0185341	PP-loop family	-0,60	-0,70
DDB0205486	putative protein serine/threonine kinase, CDK family protein kinase	0,68	0,64
DDB0218360	PhoPQ-activated pathogenicity-related protein	-0,59	-0,57
Cellular biogenesis and organization			
DDB0189693	copA, coatomer protein complex alpha subunit	0,52	0,68
DDB0216892	lvsB;"BEACH_domain-containing_protein"	1,05	0,91
DDB0202609	Transport protein particle (TRAPP)	-0,77	-0,61
DDB0187558	putative mitochondrial import inner membrane translocase	-0,65	-0,65
DDB0186481	atg9, APG9, "autophagy_protein_9"	-0,88	-0,77
DDB0217942	Putative Mpv17/PMP22 family	-0,66	-0,93
Energy			
DDB0217090	Isocitrate lyase family	-0,80	-0,84
DDB0192001	ppkA, "poly_P_kinase, polyphosphate_kinase"	-0,65	-0,51
DDB0190821	sdhB, complex II, iron-sulfur protein (IP) subunit	0,74	0,53
DDB0190821	sdhB;"complex_II,(ubiquinone), succinic_dehydrogenase"	0,59	0,61
DDB0167662	similar to Coenzyme Q9	-0,71	-0,72
DDB0204006	AMPK beta-2 chain	0,52	0,73
Metabolism			
DDB0187528	cysteine dioxygenase	0,69	0,66
DDB0185702	hgd;"homogentisate_1,2-dioxygenase"	1,05	1,10
DDB0184361	Thiamine pyrophosphate enzyme	-0,57	-0,88
DDB0186120	gshA, "gamma_glutamylcysteine_synthetase, glutamate-cysteine_ligase"	-1,72	-0,94
DDB0167249	Aldehyde dehydrogenase	-0,77	-0,94
DDB0192169	alrA;"aldehyde_reductase, aldo-keto_reductase"	-0,54	-0,88
DDB0218652	alrB;"aldo-keto_reductase"	-0,60	-0,69
DDB0189745	alrC;"aldo-keto_reductase"	-0,84	-0,72
DDB0186332	alrE;"aldo-keto_reductase"	-1,23	-0,99
DDB0204015	D-Lactate dehydrogenase	0,92	0,65
DDB0187572	Endoglucanase_E_like	0,64	0,78
DDB0203268	glgB;"1,4-alpha-glucan_branching_enzyme, branching_enzyme"	1,02	0,85
DDB0187562	glk;glucokinase	0,69	0,56
DDB0217973	Gluconolactonase	0,85	0,82
DDB0202855	Glycoside hydrolase	-1,63	-1,45
DDB0202233	Glycosyl hydrolase family 7	-1,26	-1,38
DDB0167594	Glycosyl hydrolases family	0,60	0,56
DDB0204016	gpt10;"putative_glycophosphotransferase"	-0,69	-1,11
DDB0204037	Legume lectins beta-chain signature	-0,86	-1,16
DDB0206405	Mannosyl oligosaccharide glucosidase	-0,80	-0,68
DDB0205896	NAD-dependent epimerase/dehydratase family protein	-2,12	-1,73
DDB0204752	Phosphoglycerate mutase family	0,64	0,61
DDB0190464	Predicted kinase related to galactokinase and mevalonate kinase	0,52	1,02
DDB0186919	zinc-containing alcohol dehydrogenase	-2,09	-1,99
DDB0168737	zinc-containing alcohol dehydrogenase (ADH)	-1,35	-1,03
DDB0169356	carboxylic ester hydrolase	-1,03	-0,72
DDB0190523	CAS1;"cycloartenol_synthase"	0,85	0,88
DDB0185601	cutA;"fatty_acid_elongase_3-ketoacyl-CoA_synthase, long_chain_fatty_acid_elongase"	-0,93	-0,62
DDB0188166	delta-24-sterol methyltransferase	-1,42	-1,74
DDB0186908	eapA;"alkyl-dihydroxyacetonephosphate_synthase"	0,72	0,81
DDB0187604	enoyl-CoA hydratase/isomerase domain-containing protei	-0,65	-0,63
DDB0205302	Enoyl-CoA hydratase/isomerase family	-0,64	-0,68
DDB0205157	fadB, des5-2, "delta_5_fatty_acid_desaturase"	0,89	1,11
DDB0190288	fcsB, fatty acyl-CoA synthetase, long-chain-fatty-acid-CoA ligase	-1,45	-1,57
DDB0191679	GNS1/SUR4 family	-1,03	-0,59

Table 2: Genes differentially expressed upon infection with PAO1 and PA14 versus *Klebsiella* *(Continued)*

DDB0205505	mfeB;"hypothetical_peroxisomal_multifunctional_enzyme_2"	-1,06	-0,65
DDB0191653	patatin family protein	-2,07	-1,77
DDB0218187	Perilipin family	-1,35	-1,04
DDB0184443	Saposin (B) Domains	0,59	0,59
DDB0190553	Similar to sterol-C4-methyl oxidase-like	-0,71	-0,91
DDB0217332	stearoyl-CoA desaturase	0,90	1,02
DDB0206478	allC;"allantoate_amidinohydrolase, allantoicase"	-1,12	-1,25
DDB0205700	cysteine desulfurase	-1,31	-1,26
DDB0169540	MOSC domain	-1,00	-0,67
DDB0187599	5 prime nucleotidase family	-0,90	-0,70
DDB0187063	3-methyl-2-oxobutanoate hydroxymethyltransferase	-1,83	-1,41
DDB0190860	adenine phosphoribosyltransferase	-0,98	-0,75
DDB0203073	adenosine deaminase-related growth factor	-1,35	-0,74
DDB0215237	ATP:D-ribose_5-phosphotransferase, ribokinase	-0,56	-0,51
DDB0206047	CTP synthase	-1,01	-1,20
DDB0187738	Cytidine and deoxycytidylate deaminase zinc-binding region	-0,76	-0,73
DDB0191911	putative RNA methylase	-1,01	-1,21
DDB0185785	Putative RNA methylase family	-0,59	-0,63
DDB0219236	pyrK, cytidylate kinase	-0,98	-0,71
DDB0217423	rnrB_2, ribonucleotide reductase small subunit	-0,79	-0,84
DDB0215284	tRNA/rRNA methyltransferase SpoU family protein	-0,77	-0,93
DDB0186269	Thioredoxin family	-1,41	-1,91
DDB0206431	FAD binding domain	-1,63	-1,13
DDB0187958	gchA, "GTP_cyclohydrolase_I"	-0,91	-0,89
DDB0185963	Oxysterol-binding protein	-0,55	-0,75
DDB0205608	pks18, putative polyketide synthase	0,54	0,60
DDB0168380	pks5, putative polyketide synthase	0,57	0,99
DDB0219613	stlB, putative polyketide synthase	0,68	1,05
DDB0186173	Histidine acid phosphatase	-0,79	-0,80
DDB0184156	Acetyltransferase (GNAT) family	0,81	0,78
DDB0205937	Aldehyde dehydrogenase family	1,02	0,81
DDB0183800	dihydrolipoamide_dehydrogenase	-0,60	-0,57
DDB0188526	FAD binding domain	-1,14	-0,93
DDB0169374	haloacid dehalogenase-like hydrolase	-1,26	-1,45
DDB0204714	hemA, ALAS, "5-aminolevulinate_synthase, ALA_synthase"	1,00	0,99
DDB0187575	monooxygenase, FAD-binding	-1,30	-1,14
DDB0203608	NADH:flavin oxidoreductase/NADH oxidase domain-containing protein	-2,99	-2,49
DDB0186877	Predicted hydrolases or acyltransferases	-0,70	-0,83
DDB0203708	Putative dehydrogenase domain	0,60	0,66
DDB0186921	Putative quinone oxidoreductase	-0,68	-0,80
DDB0218378	selD, selenophosphate synthase	0,83	0,85
DDB0219578	short chain dehydrogenase	-0,56	-0,76
DDB0168766	short chain dehydrogenase	0,75	0,90
DDB0201995	Short-chain alcohol dehydrogenase	-0,64	-0,69
DDB0191047	Sucrolytic enzyme/ferredoxin homolog protei	-0,76	-0,55
DDB0205223	Ubiquinone biosynthesis protein	1,02	0,99
Movement			
DDB0190345	Actin	-0,62	-0,64
DDB0216677	tubB;"beta_tubulin"	0,78	0,66
Protein targeting			
DDB0189280	CLN3 protein;Major Facilitator Superfamily	-1,38	-0,81
DDB0186130	pigF, phosphatidylinositol glycan, class Fphosphoethanolamine N- methyltransferase family	-1,13	-1,33
DDB0187271	Protein prenyltransferase, alpha subunit	-0,69	-0,66
DDB0187195	Ubiquitin family protein	-0,68	-0,56
DDB0217546	vps13B, vacuolar protein sorting-associated protein	0,80	0,57
DDB0205767	Importin-beta N-terminal domain	-1,39	-0,91
DDB0219696	CSN3, COP9 signalosome complex subunit 3	0,86	0,56
DDB0188097	mppB;"mitochondrial_processing_peptidase_beta_subunit"	0,85	0,93
DDB0188792	npl4, nuclear protein localization 4	-1,46	-1,26
DDB0189322	Peptidase family M41	0,66	0,82
DDB0204548	putative E3 ubiquitin ligase	0,56	0,88
DDB0203213	Putative serine protease	0,57	0,64
DDB0190305	RING-finger-containing ubiquitin ligase	-1,71	-2,23

Table 2: Genes differentially expressed upon infection with PAO1 and PA14 versus *Klebsiella* *(Continued)*

DDB0191910	sigB, GP63, orfGP63", "leishmanolysin_family_protein, peptidase	-1,44	-1,19
DDB0219558	usp12, putative ubiquitin carboxyl-terminal hydrolase (UCH)	0,81	0,50
DDB0188490	usp40, putative ubiquitin carboxyl-terminal hydrolase (UCH)	0,83	0,60
Signal transduction			
DDB0167328	ArfGAP, Arf GTPase activating protein	0,58	0,67
DDB0187828	gacX, RacGAP	0,62	0,52
DDB0217797	gpaG, "G-protein_subunit_alpha_7"	-0,93	-0,77
DDB0205484	GTPase-activator protein for Ras-like	0,58	0,69
DDB0202545	rabX;"Rab_GTPase"	-0,59	-0,76
DDB0186244	abkD, AdckB2, "putative_ABC1_family_protein_kinase"	0,80	0,88
DDB0217600	nek3, putative protein serine/threonine kinase	0,55	0,56
DDB0203684	tyrosine kinase-like	0,53	0,56
DDB0189806	vwkA;"protein_serine/threonine_kinase"	-0,77	-0,67
DDB0205355	Calcineurin-like phosphoesterase	-1,27	-0,86
DDB0218779	pdsA, "PDE, pde1, pdeA", "cAMP_phosphodiesterase"	0,79	1,40
DDB0204820	rabS;"Rab_GTPase"	-0,80	-0,87
DDB0190872	NLI interacting factor-like phosphatase	-0,55	-0,60
DDB0185382	Protein phosphatase 5, catalytic subunit	-0,68	-0,62
DDB0186390	protein tyrosine phosphatase	-0,85	-0,74
DDB0218065	ptpB, DdPTPa, "phosphotyrosine_phosphatase_ptp2	-1,20	-1,01
DDB0189698	Tyrosine specific protein phosphatases family	-0,76	-0,89
DDB0203756	G-protein-coupled receptor (GPCR) family protein	0,69	0,99
DDB0189216	gacD, RacGAP	-0,63	-0,58
DDB0217433	Regulator of G protein signaling	0,90	0,72
DDB0169375	cGMP-specific phosphodiesterase	-0,83	-0,79
DDB0167494	plbF, PLB, "phospholipase_B-like"	-1,17	-1,01
DDB0168860	sgkA, "SK, SPHK", "sphingosine_kinase"	-0,97	-0,79
DDB0167227	Cytochrome b5-like Heme/Steroid binding domain	-0,71	-1,57
DDB0216720	Tetraspanin family	-0,73	-0,68
Stress response			
DDB0202483	AhpC/TSA family	-0,51	-0,68
DDB0203727	AhpC/TSA family protein. Thioredoxin-like	-1,25	-1,89
DDB0203727	AhpC/TSA family protein. Thioredoxin-like	-1,11	-2,00
DDB0205904	AhpC/TSA family protein. Thioredoxin-like	-1,10	-1,63
DDB0168230	Cytochrome P450	0,61	0,67
DDB0217979	cytochrome P450 family protein	-1,11	-1,24
DDB0187276	cytochrome P450 family protein	-0,73	-0,88
DDB0186118	cytochrome P450 family protein	-0,54	-0,51
DDB0167587	Glutathione S-transferase	-2,24	-2,02
DDB0218804	Glutathione S-transferase	-1,07	-0,86
DDB0185602	putative FMN-dependent NAD(P)H:quinone reductase	-0,66	-0,93
DDB0168563	putative glutathione S-transferase	-0,64	-1,23
DDB0201962	Ku70-binding family protein	-0,60	-0,51
DDB0191833	TFIIH4, "TFIIH_subunit, general_transcription_factor_IIH,_polypeptide_4"	-0,81	-0,90
DDB0204089	NUDIX hydrolase family	-0,89	-1,14
DDB0185428	Strictosidine synthase	-1,74	-1,01
DDB0169033	trap1, Dd-trap1, "TNF_receptor-associated_protein", member of the HSP90 fam	-1,93	-1,56
DDB0188234	Chaperone clpB	-1,00	-1,25
DDB0192088	heat shock cognate protein	-0,82	-0,99
DDB0192086	heat shock protein, 70 kDa heat shock protein	-0,94	-1,18
DDB0169051	Hsp20/alpha crystallin family	-1,13	-1,30
DDB0169044	Hsp20/alpha crystallin family	-1,07	-1,12
DDB0169207	hspG12, heat shock protein Hsp20 domain-containing protein	-0,72	-0,86
Transcription			
DDB0204405	CRTF;"transcription_factor"	0,97	1,15
DDB0167879	IWS1 C-terminus	0,70	0,69
DDB0205969	snd1, tudor domain-containing protein	0,66	0,79
DDB0188840	TFIIAL, "transcription_factor_IIA"	-0,79	-0,55
DDB0167865	ddx52, DEAD/DEAH box helicase	-0,55	-0,55
DDB0184074	ddx6, DEAD/DEAH box helicase	0,62	0,66
DDB0184228	DEAD/DEAH box helicase	-0,63	-0,60
DDB0206136	myb domain-containing protein	0,59	0,58
DDB0189583	rpmA;"DNA-dependent_RNA_polymerase"	-1,33	-1,17

Table 2: Genes differentially expressed upon infection with PAO1 and PA14 versus *Klebsiella* *(Continued)*

DDB0186101	pwp2, ortholog of H. sapiens and S. cerevisiae PWP2	-0,80	-0,88
DDB0192008	rpa2, RNA polymerase I, second largest subunit	-0,98	-1,13
DDB0218008	rpc4;"putative_RNA_polymerase_III_subunit"	-0,58	-0,55
DDB0216877	tRNA pseudouridine synthase	-0,56	-0,65
DDB0204724	DNA helicase TIP49, TBP-interacting protei	0,63	0,64
DDB0219410	pirin-like protein	-0,99	-0,81
Translation			
DDB0167043	Ribosomal protein L10	-0,86	-1,37
DDB0190639	Fibrillarin	-0,65	-0,84
DDB0184302	Mitochondrial small ribosomal subunit Rsm22	-0,70	-0,72
DDB0205674	MPP10, U3 small nucleolar ribonucleoprotein	-0,77	-0,66
DDB0201601	mrpl11, S60 ribosomal protein L11, mitochondrial	-0,91	-1,26
DDB0204554	Ribosomal protein L28	-0,50	-0,69
DDB0188692	Ribosomal protein S8e	-0,94	-0,79
DDB0183814	Ribosomal RNA processing protein 4	0,58	0,56
DDB0188661	rps9, "rp1024, v12", "40S_ribosomal_protein_S9	0,60	0,70
DDB0219852	u3 small nucleolar RNA interacting protein 2, putative	-0,75	-0,63
DDB0191852	eukaryotic translation initiation factor 3 subunit 5	0,59	0,54
DDB0203843	Eukaryotic translation initiation factor 6 (EIF-6)-like protein	-0,96	-0,64
DDB0189529	gfm2, mitochondrial translation elongation factor G	-0,59	-0,50
DDB0202851	NMD3 family	-0,95	-0,97
DDB0168814	aspartyl-tRNA_synthetase	0,70	0,98
Transport			
DDB0217304	ABC transporter AbcG17	-1,58	-1,44
DDB0167281	ABC transporter mdrA2	-2,65	-1,44
DDB0167281	ABC transporter mdrA2	-2,26	-1,45
DDB0191940	abcB2;"ABC_transporter_B_family_protein"	0,87	0,93
DDB0188931	abcE1;"RNaseL_inhibitor-like_protein, non-transporter_ABC_protein"	0,61	0,74
DDB0189332	amino acid permease family protein	-0,72	-0,87
DDB0168564	Amino acid/polyamine transporter	-1,28	-1,06
DDB0190286	mcfF, Mitochondrial carrier protein	-0,99	-0,86
DDB0216936	mftA;"carrier_protein_RIM"	-0,91	-1,05
DDB0188529	nucleoporin family protein	-0,94	-0,72
DDB0189222	ccsA, copper chaperone for superoxide dismutase	-0,69	-0,62
DDB0205129	Co/Zn/Cd efflux system component	-0,56	-0,71
DDB0202441	nhe1, DdNHE1, "Na-H_exchanger, sodium/hydrogen_exchanger"	-0,77	-0,59
DDB0168533	porA;porin	0,68	0,59
DDB0218156	P-type cation-transporting ATPase	-0,69	-0,57
DDB0189480	mcfT, mitochondrial substrate carrier family protein	-0,74	-0,86
DDB0202337	Nodulin, Major Facilitator Superfamily	-0,60	-0,68
DDB0185520	Nucleoside transporter	-0,53	-0,56
DDB0203447	Sugar (and other) transporter	1,01	0,71
DDB0168979	Sugar transport proteins signature 1	-0,78	-1,28
DDB0189650	sodium/potassium-transporting ATPase alpha chain 2	-1,48	-0,81
DDB0190036	Major Facilitator Superfamily	-1,13	-1,05
DDB0205693	Major Facilitator Superfamily	-1,33	-1,08

activity is believed to be necessary to avoid the growth of intracellular pathogens and might also contribute to the efficient killing of other bacterial pathogens.

The variety of genes whose expression is altered by *P. aeruginosa* infection suggests a complex scenario in which a combined downregulation of the expression of some of the mentioned genes might affect *D. discoideum* fitness thus favoring the infection. The precise role of these genes in the pathogenesis and the mechanisms that regulates their expression will promote further investigation.

Conclusion

Our results showed that *P. aeruginosa* PA14 is more virulent than PAO1 in the *D. discoideum* model using different plating assays. The transcriptional responses of *D. discoideum* infected by either *P. aeruginosa* PAO1 or PA14 were analyzed by whole-genome microarrays and the expression of 364 genes changed similarly upon infection with

Table 3: Genes differentially expressed upon infection with PAO1 versus PA14

Gene ID	Gene function and name	Log2 ratio: PAO1-14	PAO1-C	PA14-C
Cellular Biogenesis and organization				
DDB0187116	vps13A, vacuolar protein sorting-associated protein	0,81	0,67	-0,13
DDB0189855	vps46, Vacuolar Protein Sorting	0,83	0,59	-0,24
Energy				
DDB0204335	cxgE, "cox7E, coxVIIe", "cytochrome_c_oxidase_subunit_VII_E"	1, 20	0,80	-0,40
Metabolism				
DDB0190752	diaminopimelate epimerase	1,08	0,02	-1,06
DDB0204319	Hydroxymethyltransferase	0,63	0,43	-0,20
DDB0168738	putative arginine deiminase	-0,62	-0,78	-0,16
DDB0205389	acly, ATP citrate lyase	0,89	0,75	-0,15
DDB0169357	methylenetetrahydrofolate dehydrogenase	0,78	0,21	-0,57
DDB0187393	NAD-dependent epimerase/dehydratase family protein	-0,91	-1,18	-0,28
DDB0205386	putative ATP citrate synthase	0,96	0,79	-0,17
DDB0205339	rpe;"ribulose_phosphate_3-epimerase"	0,78	0,43	-0,35
DDB0187942	Short-chain alcohol dehydrogenase of unknown specificit	0,70	0,43	-0,27
DDB0187544	smlA	1,43	1,00	-0,43
DDB0217455	zinc-containing alcohol dehydrogenase (ADH)	-1,21	-1,16	0,06
DDB0217374	zinc-containing alcohol dehydrogenase (ADH)	-1,02	-1,37	-0,35
DDB0190948	acid ceramidase-like protein	-0,87	-0,31	0,56
DDB0188248	Acyltransferase	0,64	0,16	-0,48
DDB0219652	cinB, "esterase/lipase/thioesterase_domain-containing_protein	0,92	1,28	0,36
DDB0189754	esterase/lipase/thioesterase domain-containing protein	1,07	1,07	0,00
DDB0185740	esterase/lipase/thioesterase domain-containing protein	1,08	1,27	0,20
DDB0184141	Phosphate acyltransferases	0,55	-0,26	-0,82
DDB0167446	pks16, putative fatty acid synthase	1,19	1,81	0,63
DDB0189182	Putative esterase/lipase/thioesterase	0,99	1,43	0,44
DDB0191907	dUTP diphosphatase	0,61	0,14	-0,47
DDB0217901	purH, AICAR transformylase/IMP cyclohydrolase	0,89	0,34	-0,55
DDB0167009	pyr4;"dihydroorotate_dehydrogenase, dihydroorotate_oxidase"	0,68	0,18	-0,50
DDB0217842	rpiA;"ribose-5-phosphate_isomerase"	0,63	0,42	-0,21
DDB0189571	Sulfite reductase, alpha subunit (flavoprotein)	-0,89	-0,09	0,79
DDB0202318	Cyclopropyl sterol isomerase	0,73	0,31	-0,42
DDB0167227	Cytochrome b5-like Heme/Steroid binding domain	0,86	-0,71	-1,57
DDB0168923	dihydropteridine reductase	0,60	0,14	-0,47
DDB0185998	ERG24, Ergosterol biosynthesis	0,59	-0,02	-0,61
DDB0219255	Fe(II) oxygenase superfamily	1,14	0,91	-0,23
DDB0217308	Predicted iron-dependent peroxidase	-0,55	-0,12	0,43
DDB0192180	putative O-methyltransferase	0,56	0,71	0,15
DDB0202301	putative SAM dependent methyltransferase	0,88	0,94	0,05
DDB0167345	short-chain dehydrogenase/reductase (SDR) family protein	0,73	0,56	-0,17
Movement				
DDB0188280	myoB, "DMIB, abmB", "myosin_IB"	-0,85	-0,30	0,55
DDB0167337	myoD, DMID, "myosin_ID_heavy_chain"	-0,81	-0,76	0,05
Multicellular organization				
DDB0216906	comC;"FIBROSURFIN_PRECURSOR"	-0,65	-0,39	0,26
Protein destination				
DDB0189735	homolog to co-chaperone p23	0,56	0,45	-0,11
DDB0187409	Acetyltransferase (GNAT) family	0,57	0,33	-0,24
DDB0189799	SET domain-containing protein	0,52	0,33	-0,19
DDB0202482	26S proteasome non-ATPase regulatory subunit 9	0,78	1,25	0,47
DDB0219654	Cysteine proteinase 1 precursor	0,70	0,61	-0,09
DDB0167298	Dipeptidyl aminopeptidase	0,51	-0,07	-0,59
DDB0190542	Probable proteasome subunit beta type 3	0,57	-0,06	-0,63
DDB0216902	prtA, M3L, "proteosomal_alpha-subunit_M3"	0,93	0,94	0,01
DDB0216901	prtB, M3R, "proteosomal_alpha-subunit_7-1"	1,37	1,30	-0,06
DDB0186869	small ubiquitin-like protein	0,61	0,33	-0,28
DDB0217344	ubiquitin-like domain containing CTD phosphatase	0,52	0,29	-0,23
Signal transduction				
DDB0169410	gpaB, "Ga2, Galpha2, gpa2", "G-protein_subunit_alpha_2"	-0,82	-0,55	0,27

Table 3: Genes differentially expressed upon infection with PAO1 versus PA14 *(Continued)*

DDB0190318	rab1C;"Rab_GTPase"	0,87	0,76	-0,10
DDB0202066	pakC, STE20 family protein kinase	-0,61	-0,18	0,43
DDB0169250	putative protein serine/threonine kinase	-0,87	-0,58	0,29
DDB0205782	roco6;"putative_protein_serine/threonine_kinase	-0,61	-0,48	0,14
DDB0167076	sepA, putative protein serine/threonine kinase	-0,57	-0,31	0,26
DDB0217465	fslH, G-protein-coupled receptor (GPCR) family protein	0,71	0,24	-0,46
DDB0205174	grlF, GABA-B receptor-like protein	-1,41	-1,54	-0,12
DDB0168770	grlJ, GABA-B receptor-like protein	-0,63	-0,96	-0,32
DDB0204083	grlL, GABA-B receptor-like protein	-1,84	-1,25	0,60
DDB0229801	grlQ, G-protein-coupled receptor (GPCR) family protein	0,77	0,98	0,21
DDB0167432	gacFF, RacGAP	-0,57	0,08	0,66
DDB0167384	putative guanine nucleotide exchange factor (GEF)	-0,70	-0,01	0,70
DDB0167541	dpoA;"prolyl_oligopeptidase"	-0,66	-0,11	0,55
Stress response and cell rescue				
DDB0218719	AhpC/TSA family protein	0,82	0,76	-0,05
DDB0205882	AhpC/TSA family protein. Thioredoxin-like	0,75	0,57	-0,18
DDB0217383	Cytochrome P450	1,16	0,42	-0,74
DDB0168563	putative glutathione S-transferase	0,59	-0,64	-1,23
DDB0167491	alyA;lysozyme	0,63	0,73	0,10
DDB0167489	alyB;lysozyme	0,69	0,75	0,07
DDB0167490	alyC;lysozyme	0,71	0,87	0,16
Transcription				
DDB0206051	member of NOD protein family	1,05	0,90	-0,15
DDB0202276	srfC;"putative_MADS-box_transcription_factor"	-0,90	-0,30	0,60
DDB0217613	wrky1;"putative_WRKY_transcription_factor"	1,04	1,11	0,07
DDB0167422	putative histone acetyltransferase	-0,64	-0,55	0,09
Translation				
DDB0201621	mrps2, ribosomal protein S2, mitochondrial	0,75	0,49	-0,27
DDB0218535	Eukaryotic elongation factor 1 (EF1) alpha subfamily	-0,83	-0,18	0,65
DDB0167339	tRNA-ribosyltransferase	-0,52	-0,56	-0,04
Transport facilitation				
DDB0187089	abcC5;"ABC_transporter_C_family_protein"	-1,12	-0,68	0,44
DDB0218568	Copper-transporting P-type ATPase	-1,14	-0,60	0,54
DDB0204460	patA, PAT1, "Ca2+-ATPase, P-type_ATPase"	-0,93	-0,15	0,78
DDB0205031	potassium channel tetramerization domain-containing protein	0,92	0,84	-0,08
DDB0217251	P-type cation-transporting ATPase	-2,09	-1,46	0,63
DDB0186223	P-type cation-transporting ATPase	-0,69	-0,40	0,29
DDB0167361	vatE;"vacuolar_H+-ATPase_E_subunit"	0,54	0,47	-0,08
DDB0217699	phospholipid-translocating P-type ATPase family protein	-0,67	-0,63	0,04
DDB0187945	Sugar (and other) transporter	-0,90	-0,78	0,11
DDB0206579	mcfQ, mitochondrial substrate carrier family protein	0,65	0,78	0,13
DDB0192172	mcfZ, mitochondrial substrate carrier family protein	0,73	0,92	0,18
DDB0183815	Mitochondrial carrier protein	0,55	0,81	0,26

any of both strains as compared with the control. Effects on metabolism, signaling, stress response and cell cycle can be inferred from the genes affected. Interestingly there were 169 genes differentially regulated between PAO1 and PA14, and this differential response might contribute to the different virulence behavior displayed by these two model strains. This is a starting point to begin to understand the complex relationships between environmental opportunistic pathogens and their natural hosts. Besides, our data support the idea that the host responses to different isolates of the same bacterial pathogen are largely different, thus indicating that the crosstalk between the pathogen and its host is more specific and more complex than previously thought.

Methods

D. discoideum growth and plating assays

Dictyostelium AX4 cells were grown axenically in HL5 medium (14 g/l tryptone, 7 g/l yeast extract, 0.35 g/l Na_2HPO_4, 1.2 g/l KH_2PO_4, 14 g/l glucose, pH 6.5) or in association with *Klebsiella aerogenes* on SM plates (10 g/l glucose, 1 g/l yeast extract, 10 g/l peptone, 1 g/l $MgSO_4 \cdot 6H_20$, 1.9 g/l KH_2PO_4, 0.6 g/l K_2HPO_4, 20 g/l agar, pH 6.5) [39].

For the nutrient SM-plating assay *Pseudomonas aeruginosa* (PAO1 and PA14) and *Klebsiella aerogenes (KA)* were grown overnight in LB. After washing, bacteria were resuspended with PDF (20 mM KCl; 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$; 1 mM $MgSO_4$; pH: 6.4) and the

optical density (OD) determined at 600 nm. After adjusting to 0.5 OD units, 300 μl of *Klebsiella* and *Pseudomonas* at the indicated proportions were plated in SM-agar plates with approximately 100 *D. discoideum* cells.

For non-nutrient plating assay bacteria were grown as before, washed and resuspended in PDF (at an OD of 15 units at 600 nm. 100 μl of bacteria at the indicated proportions were mixed with *D. discoideum* cells and deposited in a drop over a PDF-based non-nutrient agar plates.

Microarrays

Dictyostelium cells (5×10^7 cells) were deposited in 10 ml of HL5 (without antibiotics) in shaking culture and exposed during 4 hours to 1.0 OD (approximate multiplicity of infection: 1000) of *Klebsiella aerogenes* as a control or to a mixture of *Pseudomonas aeruginosa* (PAO1 and PA14) and *Klebsiella* (used in excess to provide similar conditions of food supply). The proportion of *Klebsiella* to *Pseudomonas* (either PAO1 or PA14) was 7:3. Previous experiments showed that these proportions are adequate for a clear inhibition of *Dictyostelium* growth in plating assays (see the results section). *Dictyostelium* cells were separated from the bacteria by gentle centrifugation (twice at 1000 rpm, 5 minutes) and RNA isolated by Trizol (Life Technologies) according to manufacturer's instructions. Three independent biological experiments were performed making a total of three independent treatments for each condition (*Klebsiella* control, PAO1, and PA14). RNA from the three treatments were paired in all three combinations (Control/PAO1; Control/PA14 and PAO1/PA14) and hybridized to three different arrays. The same was performed for the other two biological replicates making a total of 9 microarrays hybridized. One of the three biological replicates was hybridized in the opposite dye orientation to the other two. The arrays, and protocols for labelling, hybridisation and scanning were as previously described [40]. Background fluorescence was subtracted [41], linear models were fitted and the significance of apparent changes in expression was assessed using limma [42,43]. The data were normalised within each array with the printtip loess algorithm to counteract scanning and spatial biases, and further between each array to normalise mean absolute deviations using the 'scale' algorithm [44].

Preliminary analysis using ANOVA methods indicated that many genes had significant differences in expression between treatments, so we proceeded to examine each pairwise contrast in turn. We filtered out less reliable data by selecting genes with p-values adjusted for multiple testing [45] less than 0.05, making use of the moderated t statistics calculated by the eBayes function of limma. Since many genes passing this cutoff showed small changes in expression, we filtered further by absolute log2ratio. The commonly-used cutoff of greater than 2-fold change would have excluded a large number of genes that appeared to change in expression quite consistently, so we used the less stringent criterion of absolute log2ratio >0.5. A lower log2ratio would have included genes with differences in expression too small to be corroborated by other methods. The array design is available from ArrayExpress [46] under the accession A-SGRP-3. The array experiment was deposited in the ArrayExpress database under the accession E-TABM-464.

Quantitative PCR

The same RNA samples subjected to microarray study were used as templates for retrotranscription with High Capacity cDNA Reverse transcription kit (Applied Biosystems) using 250 ng of each RNA in a final volume of 20 μl. For each sample, a triplicated blank was used. This cDNAs served as template in the PCR reaction carried out in 7900 HT Fast Real- Time PCR System using Power Sybrgreen PCR Master Mix 2× with 300 nM oligonucleotides concentration in a final volume of 10 μl. The results were acquired with SDS 2.3 software by Applied Biosystems and handled with EXCEL software by Microsoft. A total of seven genes were studied for each sample and their amount were related to one control gene, DDB0217951, whose expression is not affected by the treatments with the different strains.

Authors' contributions

SC and JC performed the biological experiments and analyzed the data. GB, JS and AI designed and constructed the microarray, and provided informatics and analysis tools; GB contributed to the array experimental design, carried out the array experiments, and contributed to the analysis of results; RK was array project manager. JM contributed to the experimental design of the array and biological experiments. RE designed, coordinated the experiments and drafted the manuscript. All the authors read and approved the final manuscript.

Additional material

Additional file 1

Microsoft excel document containing all the results and genes contained in the microarray.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-8-109-S1.xls]

Additional file 2

Microsoft excel document containing the array data for the genes that showed differences in both PAO1 and PA14. The genes were manually annotated and filtered for p < 0.05 and log2 ratio >0.5 or <-0.5. Table 2 was obtained from these data.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-8-109-S2.xls]

Additional file 3

Microsoft excel document containing the array data and manual annotation of the genes that showed differential regulation between PAO1 and PA14. The genes were filtered for p < 0.05 and log2 ratio >0.5 or <-0.5. Table 3 was obtained from these data.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-8-109-S3.xls]

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CAPITULO 6: MIDA IS A PUTATIVE METHYLTRANSFERASE THAT IS REQUIRED FOR MITOCHONDRIAL COMPLEX I FUNCTION

Al igual que *vmp1* también *midA* fue hallado en un estudio de genómica comparativa entre humanos y *Dictyostelium discoideum*.

MidA es una proteína localizada en mitocondrias y relacionada con la función de este orgánulo; en este trabajo se caracterizó más a fondo su relación con el funcionamiento mitocondrial.

Los abordajes bioinformáticos son de tremenda ayuda a la hora de poder conocer la función de una proteína de función desconocida. La comparación a nivel de secuencia primaria de MidA no aportó ningún tipo de homología con otras proteínas que no sean sus homólogos en otras especies, sin embargo la comparación bioinformática a nivel de su posible estructura terciaria, encontró un dominio metiltransferasa en su secuencia, que coincidía con el presente en la proteína de *Rhodopseudomonas palustris* 1zkd. Esta proteína había sido cristalizada y definida como una posible metiltransferasa.

Mi trabajo consistió en la realización de una mutágenesis dirigida en MidA en dos aminoácidos supuestamente claves en la unión de S-adenosil metionina del dominio metiltransferasa identificado, las glycinas 170 y 172. Se construyó la proteína fusión de estas formas mutadas con GFP y se intentó la complementación de la cepa deficiente en MidA. La proteína MidA fusionada a GFP complementa totalmente el fenotipo de la cepa deficiente de *midA* sin embargo las formas mutantes no lo hacen, lo que implica al dominio metiltransferasa en la funcionalidad de la proteína.

MidA is a putative methyltransferase that is required for mitochondrial complex I function

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Summary

Dictyostelium and human MidA are homologous proteins that belong to a family of proteins of unknown function called DUF185. Using yeast two-hybrid screening and pull-down experiments, we showed that both proteins interact with the mitochondrial complex I subunit NDUFS2. Consistent with this, *Dictyostelium* cells lacking MidA showed a specific defect in complex I activity, and knockdown of human MidA in HEK293T cells resulted in reduced levels of assembled complex I. These results indicate a role for MidA in complex I assembly or stability. A structural bioinformatics analysis suggested the presence of a methyltransferase domain; this was further supported by site-directed mutagenesis of specific residues from the putative catalytic site. Interestingly, this complex I deficiency in a *Dictyostelium midA*$^{-}$ mutant causes a complex phenotypic outcome, which includes phototaxis and thermotaxis defects. We found that these aspects of the phenotype are mediated by a chronic activation of AMPK, revealing a possible role of AMPK signaling in complex I cytopathology.

Key words: *Dictyostelium*, Complex I, MidA, PRO1853, *C2orf56*, LOC55471, DUF185

Introduction

Mitochondrial diseases are caused by mutations that affect genes encoded in both the mitochondrial and nuclear genomes. The pathological phenotypic outcomes of mitochondrial diseases are very complex and include blindness, deafness, epilepsy, heart disease, and muscle and neurological disorders. Although much is known about the associated mutations, the relationship between genotype and phenotype is complicated and poorly understood. Surprisingly, the same genetic defect can result in different symptoms, and conversely, similar outcomes can be caused by different genetic lesions (Debray et al., 2008; DiMauro and Schon, 2008).

Among mitochondrial diseases, deficiencies in complex I (CI) are very relevant in human pathology because about 40% of mitochondrial OXPHOS diseases involve complex I defects, and the molecular cause of this deficiency is unknown in many patients (Janssen et al., 2006; Lazarou et al., 2009). Most cellular ATP is generated by the mitochondria through aerobic respiration. Together with complex III and complex IV, CI contributes to the generation of a proton gradient across the mitochondrial inner membrane. This proton gradient is used by the ATP synthase (complex V) for ATP production. Mitochondrial CI (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) is a huge multiprotein complex of 45 subunits in mammals. Of the five major respiratory complexes, CI is the least understood, partly because of its large size and complexity. Accordingly, it is believed that new components involved in the assembly, stability and/or activity of CI still remain to be identified (Koopman et al., 2010; Remacle et al., 2008).

Extensive post-translational modifications of complex I subunits have been described, most of which affect the N-termini. Examples include the loss of mitochondrial import sequences and N-alpha-acetylation. Phosphorylation in several CI subunits has also been described to affect CI function. Mutational analysis of the phosphorylation sites of NDUFA1 and NDUFB11 revealed defects in CI assembly (Koopman et al., 2010). The presence of methylation in two CI subunits has also been previously described, although the functional relevance and the methyltransferase responsible are not yet known (Carroll et al., 2005; Fearnley et al., 2007; Wu et al., 2003). In contrast to N-alpha-acetylation, which appears to be a permanent modification, and similarly to phosphorylation, protein methylation might be reversible by demethylases and might have a regulatory role.

The social amoeba *Dictyostelium discoideum* is a useful model for the study of biological issues that are relevant to human disease, including mitochondrial dysfunction (Annesley and Fisher, 2009a; Barth et al., 2007; Bokko et al., 2007; Chida et al., 2004; Kotsifas et al., 2002; Torija et al., 2006b; Williams et al., 2006). *Dictyostelium* cells feed on bacteria and remain in the form of individual cells while food is present. However, when the supply of bacteria is exhausted, starvation triggers a remarkable process of cellular chemotaxis, allowing the formation of cell aggregates. These aggregates differentiate to form phototactic migrating slugs that eventually give rise to fruiting bodies containing spores that allow *Dictyostelium* to survive (Escalante and Vicente, 2000). This developmental program is sensitive to mitochondrial dysfunction, and slug phototaxis and thermotaxis in particular are affected by diverse mitochondrial defects (Annesley and Fisher, 2009b;

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Wilczynska et al., 1997). Moreover, *Dictyostelium*, as opposed to the yeast model *Saccharomyces cerevisiae*, contains all the essential CI subunits (Eichinger et al., 2005; Ogawa et al., 2000).

Traditionally, it has been assumed that mitochondrial diseases mediate their effects on the phenotype through the reduced availability of ATP. However, recent studies in *Dictyostelium* suggest that some symptoms might be the consequence of abnormal regulation of signaling pathways. The relationship between AMP-activated protein kinase (AMPK), a master regulator of the energy status of the cell, and mitochondrial diseases has been recognized recently using *Dictyostelium* as an experimental model (Bokko et al., 2007). Previous studies suggest that a chronic activation of AMPK might be a key element in some of the observed phenotypes that are present in mitochondrial dysfunction (Barth et al., 2007).

Recently, we described the identification and initial characterization of a new mitochondrial protein conserved between *Dictyostelium* and humans that we named MidA (for mitochondrial dysfunction protein A) (Torija et al., 2006a; Torija et al., 2006b). This protein belongs to an uncharacterized conserved protein family (DUF185 or COG1565). It shows high similarity to the human protein of unknown function LOC55471 encoded on chromosome 2 (C2orf56 or PRO1853).

Dictyostelium midA^{-} cells showed reduced levels of ATP and a wide array of phenotypes, including slow growth and abnormal development. In this report, we have further analyzed the function of this mitochondrial protein using an integrated approach of bioinformatics and molecular genetics in *Dictyostelium* and human cell culture. The loss of MidA generated a mitochondrial dysfunction that specifically affected CI activity and the levels of the fully assembled complex. Moreover, both *Dictyostelium* and human MidA proteins interact with NDUFS2, an essential CI core subunit. The molecular function of MidA was studied by bioinformatics and site-directed mutagenesis. The results indicate the presence in this protein family (DUF185) of a methyltransferase fold, suggesting that methylation has an important role in CI function. The phenotypic outcome observed in the *Dictyostelium midA*$^{-}$ null mutant reveals the complexity of CI mitochondrial disease and the contribution of AMPK signaling.

Results

Dictyostelium and human MidA mitochondrial proteins are required for complex I activity

Dictyostelium and human MidA are highly homologous proteins. Our previous studies in *Dictyostelium* showed that MidA is a mitochondrial protein involved in bioenergetics and its high sequence homology among species suggested the possibility of functional conservation between humans and *Dictyostelium* (Torija et al., 2006b). We wanted to test this hypothesis and extend our knowledge of the function of these proteins. Consequently, our first aim was to determine the subcellular localization of MidA in human cells. A construct expressing the human protein fused to GFP was transiently transfected into HeLa and HEK293T cells, which were then stained with the mitochondria-specific dye Mitotracker Red. As shown in supplementary material Fig. S1, human MidA was also localized in mitochondria.

We next took advantage of comparative genomic tools to design working hypotheses about MidA function. There are homologues of MidA in many organisms but it seems to be absent in others and this phylogenetic profile might provide important functional clues. It is expected that proteins working together in a given function will have the same phylogenetic profile. Using the String server (http://string.embl.de/), we obtained very similar phylogenetic profiles for CI subunits, well known CI assembly factors and MidA. A more detailed analysis using a wide array of species is shown in supplementary material Table S1. There is a clear correlation between the presence of MidA and a representative complex I subunit (NDUFS7). It is well known that CI is not present in all eukaryotes (such as fermentative yeasts *S. cerevisiae* and *S. pombe*) but it is present in higher eukaryotes and *Dictyostelium* where it has a key role in ATP generation. As expected, MidA has no homologues in *S. cerevisiae* and *S. pombe*. Interestingly, MidA homologues are also found in α-proteobacteria, the closest living organisms to the putative precursors of eukaryote mitochondria.

To test the hypothesis of a functional connection between MidA and CI, we measured the activity of the OXPHOS complexes (I, II, III and IV) in *Dictyostelium midA*$^{-}$ null cells. A 50% decrease in the activity of CI in *Dictyostelium* was observed (Fig. 1A). Interestingly, the activity of the other complexes was either unaffected or was significantly higher in the mutant. This might be explained by a compensatory response to the loss of CI activity.

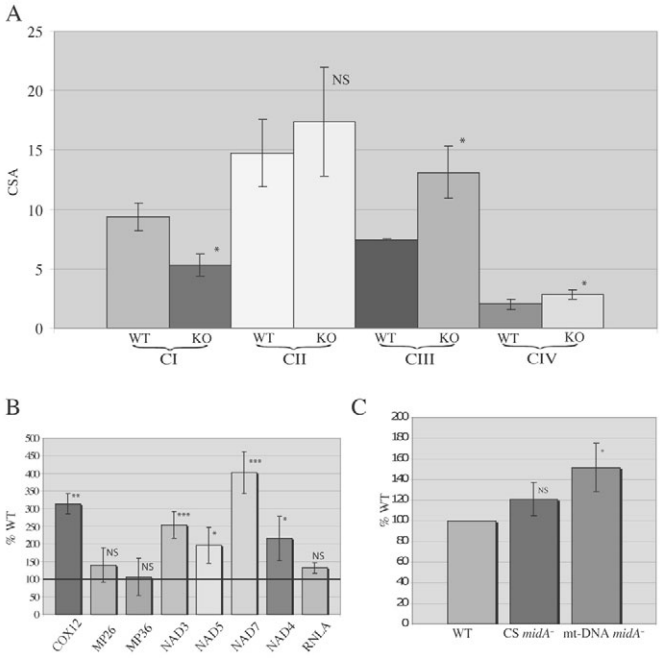

Fig. 1. The activity of complex I is reduced in cells lacking MidA. (**A**) Spectrophotometric analysis of the activity of complexes I, II, III and IV in *Dictyostelium* WT and *midA*$^{-}$ mutant cells. At least three independent experiments for each complex were performed and the bars represent mean ± s.d. Significance of differences were determined by Student's *t*-test; **P*<0.05; ***P*<0.01; ****P*<0.001; n.s., non-significant difference. Reduced activities of complex I were observed in *midA*$^{-}$ *Dictyostelium* cells. CSA, complex specific activity, corrected by citrate synthase. (**B**) The steady-state levels of expression of representative genes from the eight major polycistronic transcripts from mitochondrial RNA were measured and compared with the levels found in wild-type control cells. Five of them showed significant increases compared with wild-type cells. Three independent experiments were performed and the bars represent the mean ± s.d. Significance of differences were determined by Student's *t*-test and indicated by the *P* value, as described above. (**C**) The level of the activity of the mitochondrial enzyme citrate synthase (CS) and amount of mtDNA was measured and compared with the wild type (adjusted to 100%). Three independent experiments were performed and the statistical analysis was carried out as described above.

Consistent with this possible compensatory response, increases were also observed in the mutant in the steady state levels of mtDNA and most mtRNA transcripts (Fig. 1B,C). There also appeared to be a small, albeit statistically insignificant, increase in the activity of citrate synthase (Fig. 1C).

The level of assembled complex I is reduced in HEK293T cells where MidA is downregulated

We next wanted to study the level of assembled complex I by blue native (BN)-PAGE. We were unable to use *Dictyostelium* cells in these analyses owing to the lack of available antibodies to detect the different complexes and the low signal obtained by BN-PAGE. Therefore we used stable transfectants of HEK293T cells in which human MidA was downregulated by shRNAmir technology. Two different clones (S1 and S19) were obtained with a level of *MidA* mRNA downregulation of 93% and 87%, respectively. A control clone transformed with a scrambled vector showed no reduction in *MidA* mRNA. Fig. 2A shows representative gels and Fig. 2B a quantification by densitometry of three independent experiments where the level of CI had been normalized using the signal of complex III. Similar results were obtained when we normalized for complex II, complex IV and complex V (data not shown). We found a modest effect in fully assembled CI that ranged from 60% to 80% with respect to the control, which was treated with scrambled shRNA. The activity of CI was also measured by spectrophotometric analysis. A reduction in CI activity was only detected in clone S1, which had higher levels of mRNA inhibition. This clone showed an average CI activity that was 75% of that in control cells (data not shown). Together, these results suggest that both *Dictyostelium* and human MidA are involved in complex I assembly or stability.

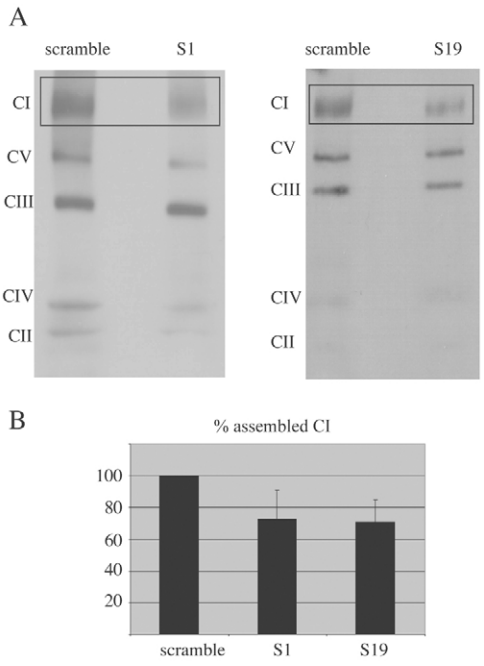

Fig. 2. Reduction of fully assembled complex I in human cells with downregulated MidA. (**A**) Mitochondrial complexes from human HEK293T clones S1 and S19, and control cells were extracted with N-dodecyl β-D-maltoside for BN-PAGE analysis. The gel was transferred to PVDF membrane and incubated with a cocktail of antibodies for complex detection. The different bands were assigned by the expected pattern and their approximate size calculated using a protein size standard. A significant reduction in CI levels (indicated by rectangle) was observed. (**B**) Densitometric quantification of three different BN-PAGE western blots showed a reduction of assembled CI to approximately 70-80% of that found in a clone harboring a scrambled vector. Densitometry was performed with ImageJ 1.33u (NIH, USA), and the values for CI were normalized to those of complex III. The bars represent mean ± s.d. P=0.060 for S1 and P=0.033 for S19 (Student's t-test).

MidA interacts with complex I subunit NDUFS2

In a parallel approach to elucidate the function of MidA, we performed a yeast two-hybrid screening to identify possible interactors. As bait we used the whole *Dictyostelium* MidA protein, except for the first 21 amino acids, which correspond to the putative mitochondrial targeting sequence. The vector used was pB29, which contains an N-bait-LexA-C fusion, and 57.4 million interactions were analyzed. Interestingly, ten independent positive clones were obtained that encode *Dictyostelium* NADH-ubiquinone oxidoreductase-chain 49 (DDB_G0294030), a homologue of the human complex I subunit NDUFS2. Fig. 3A shows the common region contained in the different clones that allowed us to restrain the minimal interaction region to 40 amino acids. This interaction was ranked with high confidence by a computer program (Global PBS®, Predicted Biological Score from Hybrigenics) that represents the probability of an interaction to be non-specific (Rain et al., 2001). Nevertheless, a further validation was performed using pull-down assays. The N-terminal fragment of *Dictyostelium* NDUFS2, which contains the minimal region of interaction, was fused to GST, purified from bacteria and incubated with cell extracts from *Dictyostelium* expressing MidA fused with GFP. As shown in Fig. 3B, MidA-GFP was pulled down by GST-NDUFS2 but not by GST alone, which was used as a control. We also wanted to validate this interaction using human MidA, expressed in HEK293T cells fused with GFP. The bacterially expressed N-terminus of human NDUFS2 was used in the assay (Fig. 3B). A positive pull-down was observed, again suggesting a functional conservation between *Dictyostelium* and human MidA proteins.

We next asked whether the observed interaction requires the functional methyltransferase domain identified in the MidA sequence (see below). We performed pull-down assays using *Dictyostelium* cell extracts expressing wild-type and mutated MidA (G170V) in which the methyltransferase domain is presumed to be inactivated (see below). As shown in Fig. 3C, both proteins were pulled down by the bacterially expressed *Dictyostelium* NDUFS2, suggesting that a functional methyltransferase domain is not required for the interaction.

Is MidA stably bound to CI or any other large subcomplex? To answer this question, we used the same *Dictyostelium* rescued strain used in the pull-down experiments that stably expressed MidA-GFP to perform a second-dimension analysis. After BN-PAGE, the lane was cut and resolved in a second SDS-denaturing dimension and MidA-GFP was detected by western blot analysis (supplementary material Fig. 2B). MidA had an apparent molecular size in the first dimension that was compatible with it being a homodimer (approximately 200 kDa, after taking into account the size of the GFP and TAP tags). Although most of the MidA protein is present as a dimer, a signal was also detected at higher molecular sizes (ranging from 450 to 750 kDa) (supplementary material Fig. S2B). *Dictyostelium* CI has an approximate molecular weight of 880 kDa, as determined previously by MALDI-TOF analysis of the corresponding band from BN-PAGE gels (supplementary material Fig. S2A). Therefore, *Dictyostelium* MidA does not seem

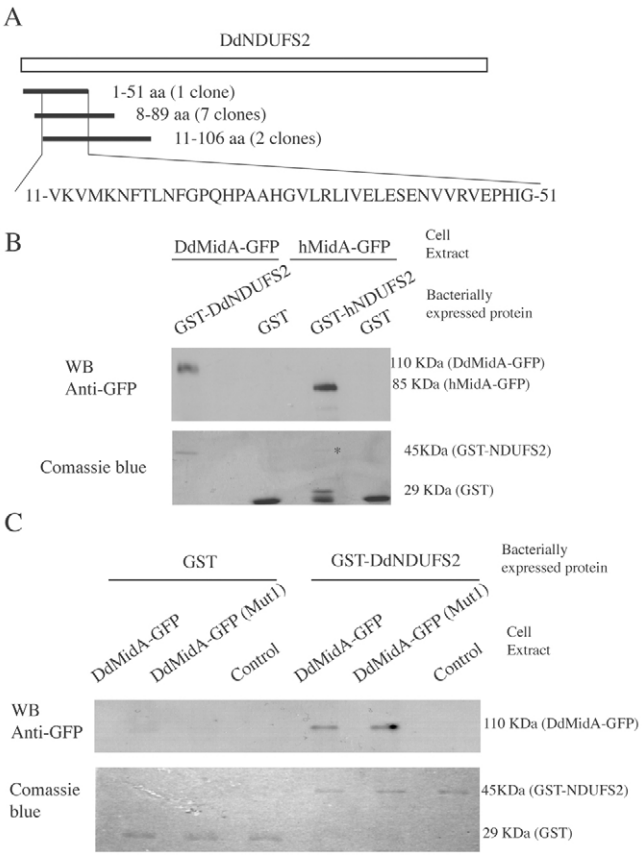

Fig. 3. MidA interacts with NDUFS2. (**A**) A yeast two-hybrid screening using *Dictyostelium* MidA as bait rendered a possible interaction with the N-terminus of the complex I core subunit NDUFS2. The open rectangle represents the complete amino acid sequence of *Dictyostelium* NDUFS2 and the lines below mark the position and the number of the different clones that gave a positive interaction in the screening. The amino acid sequence of the overlapping minimal region is also shown. (**B**) The interaction was validated by pull-down assay using bacterially expressed NDUFS2 fused with GST and cell extracts expressing *Dictyostelium* MidA and human MidA fused to GFP. A fraction of the bacterially expressed proteins used in the assay are shown by Coomassie blue staining after SDS-PAGE electrophoresis. Human NDUFS2 was sensitive to degradation and little protein was obtained (labeled with an asterisk). After incubation with the indicated cell extracts and subsequent washes, the samples were separated by SDS-PAGE, transferred and incubated with anti-GFP antibody. Both *Dictyostelium* and human MidA-GFP were efficiently pulled down and showed the expected molecular size. (**C**) Similar pull-down assays were performed using *Dictyostelium* cell extracts expressing MidA-GFP as before, or a mutated form (G170V), indicated as MidA-GFP (Mut1). A wild-type extract was used as an additional negative control. The lower blot shows the bacterially expressed proteins used in the assay stained with Coomassie blue. The upper blot shows both proteins interacting with NDUFS2.

to be stably bound to CI, but might be present in high molecular mass CI subcomplexes. This is in agreement with previous proteomic studies that have not detected MidA as part of CI (Fearnley et al., 2007). A similar experiment was performed with HEK293T expressing human MidA-GFP and we found no stable association of the human protein with subcomplexes, although its size suggested that it is also a homodimer (data not shown). Perhaps the interaction of the *Dictyostelium* protein in large complexes is more stable than that of the human homologue, allowing its detection by this technique, or there might be functional differences between species that we do not yet understand.

MidA has a conserved methyltransferase fold

The lack of obvious functional motifs in MidA amino acid sequences prevented us from speculating about the possible molecular function (Torija et al., 2006b). We have now searched for structural similarities with other proteins whose function is known and carried out bioinformatics modeling. To obtain a structural model for MidA, we performed an initial Blast search against PDB, the database of protein structures, to find a homologous protein of known structure (Altschul et al., 1997; Kirchmair et al., 2008). We found a highly similar candidate with 32% sequence identity with MidA and a Blast E-value of 2e-52. This protein belongs to *Rhodopseudomonas palustris* and is a target of a structural genomics consortium (Uniprot code Q6N1P6, PDB code: 1zkd). Its function is still unknown, as shown by its placement in the PFAM database of conserved domains as a DUF185 member, a family of proteins of unknown function (Finn et al., 2008). However, in the SCOP database of the structural classification of proteins (Andreeva et al., 2008), 1zkd was classified as a possible member of the S-adenosyl-L-methionine (SAM)-dependent methyltransferases, a large superfamily of proteins that is comprised of 52 protein families.

Using 1zkd as a template, a 3D model of MidA was built using the Phyre server (Kelley and Sternberg, 2009). From the set of models returned by this server, the one at the top of the list uses 1zkd as template, reflecting once more the relatedness of the two proteins. The sequence-to-structure alignment between MidA and 1zkd covered most of the MidA sequence (Fig. 4A). Of course, the MidA N-terminal sequence corresponding to the putative mitochondrial-targeting peptide, is totally excluded in the model.

We next wanted to define the functional residues that might occur in the catalytic site using FireStar (Lopez et al., 2007), a web server that predicts functionally important residues using FireDB (Lopez et al., 2007). FireDB is a database of PDB structures and their associated ligands, and contains the largest set of reliably annotated functionally important residues. These annotations are extracted from protein-ligand atom contacts and are also derived from the catalytic-site atlas (CSA) (Porter et al., 2004). Interestingly, the information obtained from FireStar indicated a relationship between the sequence of MidA and a human dimethyladenosine transferase (PDB code 1zq9, chain A). Following the FireStar annotations derived from the CSA, the catalytic site of 1zq9 contains three relevant residues: G64, E85 and N128. The two first are conserved in MidA (G170 and E200), whereas the third is replaced by a glutamine (Q257). The structure of the human dimethyladenosine transferase (1zq9) is available (PDB code 1zq9, chain A) and lzq9 was crystallized with S-adenosylmethionine (SAM), the methyl donor, that was in contact with these three residues. We superimposed our MidA 3D model with the region that contains the 1zq9 catalytic site (residues 64-128) using local-global alignment (LGA) (Zemla, 2003), and found that they superimposed fairly well, as shown in Fig. 4B.

To determine whether the SAM ligand can be accommodated in our MidA model in the proper manner, a docking was performed using the Haddock biomolecular docking software by searching 1000 models. The only spatial restriction imposed on Haddock was to keep SAM close to the equivalent three residues in our model. We obtained several clusters of models, and finally selected the first ten models of the best cluster. These models had good

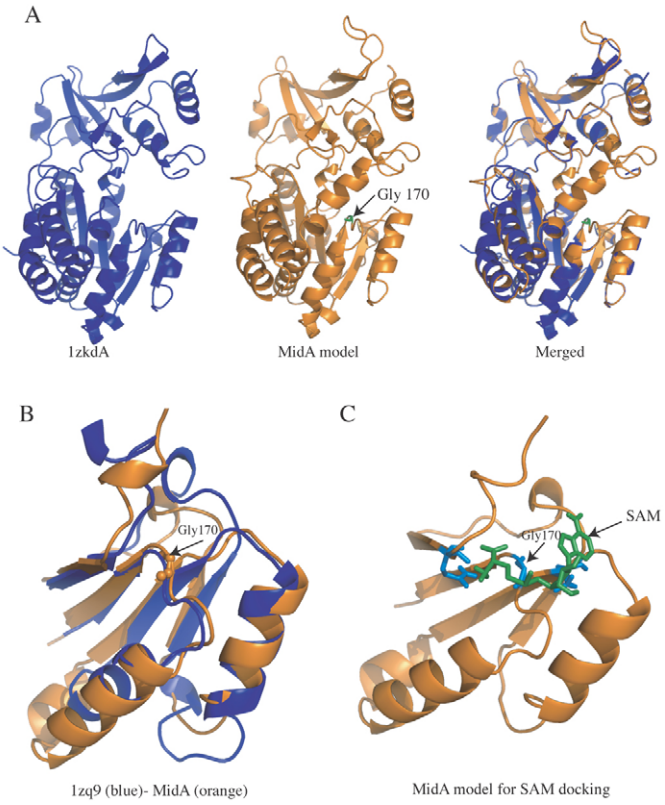

Fig. 4. MidA contains a methyltransferase domain. (**A**) A 3D model of MidA was built using as a template 1zkd, a *Rhodopseudomonas palustris* protein that belongs to DUF185 family. The blue diagram represents 1zkd, chain A from PDB (this protein is a homodimer and chain B is not displayed). The orange diagram shows the MidA model. The structural alignment between MidA and 1zkd is shown on the right and covers most of the MidA sequence. (**B**) Detail of the alignment of the MidA model and 1zq9. The catalytic region of the 3D model of MidA (orange) superimposes fairly well with that of 1zq9 (blue), a human dimethyladenosine transferase whose structure is known, showing a characteristic fold exposing a loop where several conserved residues reside. Among them, Gly170, was chosen for functional analysis. (**C**) The methyl donor SAM fits in the MidA model. Docking was performed running Haddock. SAM maintains the correct distances to the equivalent critical residues of the methyltransferase 1zq9. Residues G170, E200 and Q257 are blue and SAM is colored in green.

Haddock scores and were very similar, with a maximum interface ligand RMSD of 1.5 Å. The first ranked model is shown in Fig. 4C. The network of contacts between MidA and SAM remained, as shown with Ligplot (Wallace et al., 1995) in supplementary material Fig. S3, keeping the three aforementioned residues in contact with SAM.

The next step was to simulate the effect of the mutation G170V. We first replaced G170 with a valine with PyMol over the docking model (The PyMol Molecular Graphics System, DeLano Scientific, Palo Alto, CA) by selecting the rotamer that appears to be more frequent in proteins. Obviously, the effect of this mutation produced new clashes and so required a new docking of SAM with respect to the mutated model. We ran Haddock again to better accommodate the SAM molecule. Once more, we picked the first ten models of the best cluster, and checked the level of conservation of the spatial distance with respect to the three aforementioned residues in the mutation model. The new docking model allowed us to determine that the initial network of contacts was lost, and that SAM was now accommodated towards the exterior of the protein, presumably causing a loss of the function of the enzyme. This was confirmed experimentally by site-directed mutagenesis, as described below.

The crystal structure of 1zq9 indicates that the protein is homodimeric, as described for other methyltransferases. It should be noted that the BN- and SDS-PAGE analysis described above suggested that MidA is also a dimer (supplementary material Fig. S2).

The methyltransferase domain is required for MidA function

The structural model shown above strongly suggested the presence of a SAM-binding motif, which is characteristic of methyltransferases. To confirm this hypothesis, we performed site-directed mutagenesis to change residue G170, which is present in the conserved folding and whose change to valine was predicted to have a deleterious effect on the binding of SAM. A double mutation was also performed to change both G170 and G172 to valines. Both residues have been previously suggested to be important for SAM binding, and are well conserved among methyltransferases (Niewmierzycka and Clarke, 1999). The WT and mutated forms of MidA were fused to GFP to monitor their expression and localization in the cells and transformed into the *Dictyostelium midA*$^{-}$ null mutant (Fig. 5A). As described previously, *midA*$^{-}$ cells show defects in growth both in axenic medium and in association with bacteria, as a consequence of the mitochondrial dysfunction (Torija et al., 2006b). Although the WT protein complemented the phenotype completely, the G170 mutated protein was not able to complement the phenotype (Fig. 5C). A similar result was obtained for the G170 and G172 double mutation (data not shown). To exclude the possibility that the lack of complementation was a result of failed targeting to the mitochondria, we checked and showed that the proteins were localized in the mitochondria (Fig. 5B shows the result for the G170V mutant as an example). The lack of reversion of the phenotype strongly suggests that a single mutation in G170 from the methyltransferase domain is sufficient to render the protein inactive. These results also suggest that the putative methyltransferase domain is required for MidA function in *Dictyostelium*.

Characterization of MidA complex I deficiency and its relationship with AMPK signaling

To gain a better understanding of the genotype-phenotype relationship in mitochondrial dysfunction, we made use of *Dictyostelium* cells lacking MidA (*midA*$^{-}$ null mutant) as a cellular model for CI disease. We wanted to characterize in detail the phenotype of the null mutant and to study the relationship with AMPK signaling. *Dictyostelium* cells aggregate upon starvation to form a multicellular organism. At the slug stage, these structures show a remarkable capacity to move towards light and thermal gradients. This phototaxis and thermotaxis ability was shown to be more sensitive to mitochondrial dysfunction than other cellular functions (Kotsifas et al., 2002; Wilczynska et al., 1997). The *midA*$^{-}$ mutant showed a strong defect in phototaxis, as observed by the slug-trail assay (Fig. 6A, upper panel) and quantification of the accuracy of phototaxis (κ) (Fig. 6A, lower panel). κ measures how concentrated the trails are around the direction of the light source. It ranges from 0 when there is no orientation in any preferential

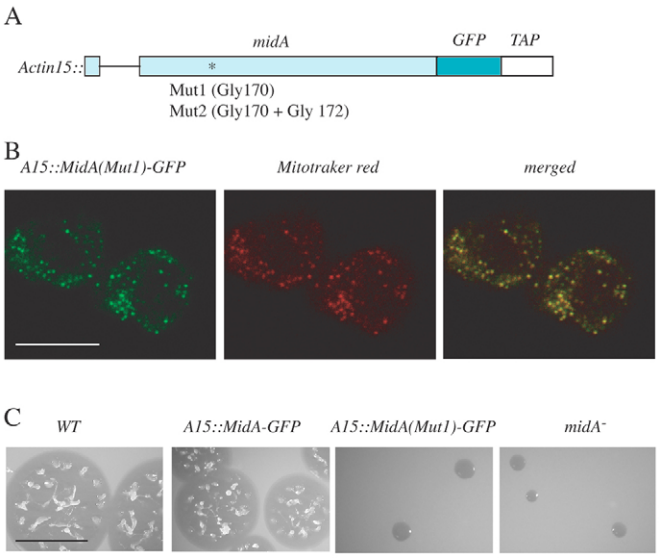

Fig. 5. Site-directed mutagenesis supports the presence of a methyltransferase domain. (**A**) Scheme of the expression constructs used for complementation studies. The coding region of the *midA* gene, fused to GFP and TAP (tandem affinity purification tag), is directed by the Actin15 (A15) promoter and is depicted as open boxes. A thin line represents an intron near the N-terminus. Site-directed mutagenesis was performed at the indicated residues (asterisk). (**B**) The wild-type and mutated constructs were transformed in the *midA*$^{-}$ mutant and stable transformant clones were checked for expression and localization of MidA in mitochondria. All the constructs showed mitochondrial localization of the protein. A representative analysis is shown for the mutated construct (G170V). Colocalization was observed between MidA-GFP (green) and mitotracker (red), indicating mitochondrial localization. Scale bar: 10 µm. (**C**) Analysis of growth in association with bacteria of transformant clones with WT and the mutated forms of MidA. The WT construct complemented the growth phenotype and other aspects of the phenotype (not shown). However, the constructs containing the indicated mutations were not able to complement the phenotype, giving rise to the small colony phenotype that is characteristic of *midA*$^{-}$ mutant. A representative analysis is shown for the mutation G170V. Scale bar: 1 cm.

direction to infinity in the case of a perfect orientation. The defect in phototaxis was restored in a *midA*$^{-}$ mutant strain where *Dictyostelium* MidA had been transformed with an expression vector under the control of an actin promoter (rescued strain). Similarly, a defect in thermotaxis was also observed, as shown in Fig. 6B where κ was measured in the wild type, the *midA*$^{-}$ mutant strain and the rescued strain during thermotaxis at different temperatures (Fig. 6B). Thermotaxis was also complemented in the rescued strain. Additionally, *midA*$^{-}$ mutants showed a growth defect that is accompanied by a phagocytosis and macropinocytosis defect, as previously described (Torija et al., 2006b) and shown in supplementary material Fig. S6. These defects in phagocytosis and macropinocytosis have not been observed previously in other mitochondrial *Dictyostelium* mutants, suggesting a more complex scenario, which will be discussed below (Barth et al., 2007).

We next explored the contribution of AMPK signaling in the complex phenotype of *midA*$^{-}$ cells. Overexpression of an AMPK antisense construct has been previously shown to inhibit the expression of AMPK and to restore phototaxis in several *Dictyostelium* mutants. As expected, we found that phototaxis and partly thermotaxis were restored in the *midA*$^{-}$ mutant when AMPK was downregulated suggesting that this phenotype is mediated by

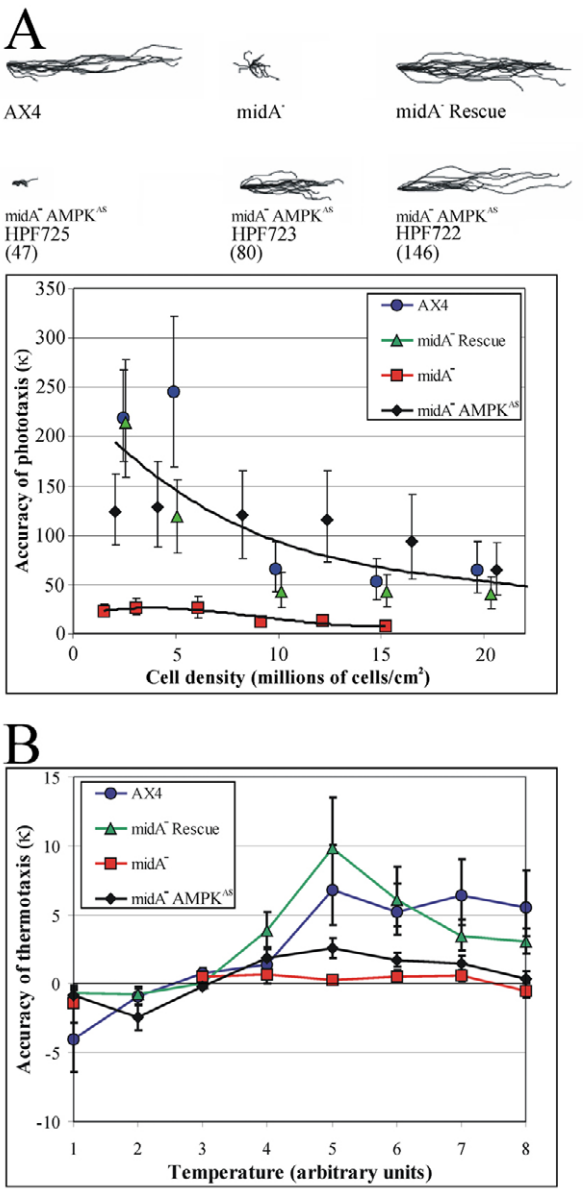

Fig. 6. Phototaxis defects in *Dictyostelium* MidA mutant can be rescued by AMPK inhibition. (**A**) Different *Dictyostelium* strains were allowed to form migrating slugs and exposed to lateral light to determine their phototaxis capabilities. The strains used were the following: wild-type *Dictyostelium* cells (AX4), the *midA*-null mutant (*midA*$^{-}$), a complemented strain ectopically expressing the complete *midA* gene (*midA*$^{-}$ Rescue) and several independent transformants (strain designations beginning with HPF) of the MidA mutant expressing different numbers of copies of an AMPK antisense construct (*midA*$^{-}$ AMPK^{AS}). The copy number for the AMPK antisense construct (pPROF362) is indicated within brackets. The phototaxis and migration (short trails) defects of the *midA*$^{-}$ mutant were suppressed in a copy number-dependent manner. In the lower panel, the accuracy of phototaxis (κ) was measured for the indicated strains at different cell densities. In this experiment, the *midA*$^{-}$ AMPK^{AS} strain was HPF722. (**B**) Defective thermotaxis in the *midA*$^{-}$ mutant is complemented by ectopic expression of MidA and suppressed by AMPK antisense inhibition. The accuracy of thermotaxis in a 0.2°C/cm gradient was measured for slugs formed at a density of 3×10^{6} cells/cm^{2} and migrating at temperatures ranging from 1 to 8 (arbitrary units corresponding in separate calibration experiments to 14°C to 28°C). The *midA*$^{-}$ AMPK^{AS} strain was HPF723 and it exhibited substantial (but not complete) suppression of the thermotaxis defect. Other details are as for A.

a chronic activation of the kinase (Fig. 6A). This is the first report that a phenotypic defect caused by a specific CI deficiency in a model system can be rescued by AMPK downregulation. Interestingly, the defects in growth, phagocytosis and macropinocytosis were not rescued by the same experimental manipulation, suggesting that these defects are independent of AMPK signaling in the *midA*$^{-}$ mutant (supplementary material Fig. S4).

Discussion

We used bioinformatics and experimental comparative analysis in *Dictyostelium* and human cells to shed light on the function of MidA, a protein that is conserved from bacteria to humans and contains a characteristic DUF185 motif of unknown function. The functional similarities between *Dictyostelium* MidA and its human homologue strongly suggest that both proteins are orthologous proteins that contain a methyltransferase domain and required for mitochondrial CI function. Another putative methyltransferase has recently been described to function in CI assembly or stability (Gerards et al., 2009; Sugiana et al., 2008), highlighting the potentially important but poorly understood role of methylation in CI function.

About 40% of inherited disorders of the OXPHOS system involve isolated or combined deficiencies in CI, the largest complex of the OXPHOS system. The genetic cause of many cases of CI deficiencies is still unknown, which is due in part to insufficient understanding of the CI assembly process and the factors involved. We do not know yet whether MidA is involved in human mitochondrial disorders, but it should definitely be considered a strong candidate. The loss of function of this protein in the model *Dictyostelium* generates a complex phenotypic outcome, including growth and developmental defects. In humans, disorders associated with CI dysfunction are also complex, and usually lead to multisystem failure that affects the brain, skeletal muscle and heart.

Dictyostelium midA^{-} null cells showed a decreased activity of CI, and BN-PAGE studies in human cells where MidA is downregulated also showed lower amounts of fully assembled complex, suggesting a role for MidA as an assembly or stability factor. The level of the assembly and activity of human CI in the knockdown cells was around 70% of the control values, a moderate effect but near the threshold that could have an impact in cellular bioenergetics (Pathak and Davey, 2008), or even cause human disease (Loeffen et al., 2001). CI is formed by a large number of subunits (up to 45 subunits in humans). The assembly and stability of such a large multiprotein complex requires specific chaperone and assembly factors, six of which have been implicated in human CI deficiency, including NDUFAF1-NDUFAF4 (Hoefs et al., 2009; Ogilvie et al., 2005; Saada et al., 2009; Vogel et al., 2005), C8ORF38 (Pagliarini et al., 2008) and C20ORF7 (Gerards et al., 2009; Sugiana et al., 2008). Others such as Ecsit (Vogel et al., 2007), AIF (Vahsen et al., 2004) and IndI (Bych et al., 2008) are required for CI assembly, but have not yet been implicated in human disease.

In spite of its large size, CI has a basic catalytic core formed by only 14 proteins that are evolutionarily conserved from prokaryotes to humans. All have been implicated in human CI disorders, including NDUFS2 (Loeffen et al., 2001; Ugalde et al., 2004), an iron-sulfur protein that we have shown to interact with MidA/PRO1853. NDUFS2 is encoded by the nuclear genome of human cells but it is encoded by the mitochondrial genome in *Dictyostelium* revealing its ancient origin as an endosymbiont protein. Interestingly, MidA homologues also exist in α-proteobacteria, suggesting that the interplay between MidA protein and the catalytic CI is of ancient origin and conserved from bacteria to humans.

The SAM-dependent methyltransferase enzymes share little sequence identity, but do contain a highly conserved structural fold that is involved in SAM binding. Surprisingly, the precise residues that bind the SAM cofactor are poorly conserved (Loenen, 2006; Schubert et al., 2003). We have shown by bioinformatics modeling and site-directed mutagenesis that DUF185 might have a methyltransferase domain. At the level of amino acid sequence, only a short region of homology can be detected between DUF185 proteins and methyltranferases, the so-called motif I, corresponding to a loop of the catalytic core involved in SAM binding (Niewmierzycka and Clarke, 1999). The consensus sequence was defined as a nine-residue block, hh(D/E)hGxGxG, where h represents a hydrophobic residue and x can be any residue (Kakebeeke et al., 1979; Niewmierzycka and Clarke, 1999). In previous studies based on multiple protein alignments, this short sequence was revealed and led to the proposal that DUF185 is a methyltransferase (Sadreyev et al., 2003). Our site-directed mutagenesis studies targeted the first two conserved glycines present in this sequence and showed that the first and possibly both of these residues are required for the protein function. Five different structural folds have been described to bind SAM and perform a methyl transfer. Our model fits with class I, the most abundant, which is composed of alternating β-strands and α-helices (Martin and McMillan, 2002; Schubert et al., 2003). Taken together, these studies strongly suggest that MidA proteins contain a methyltransferase domain. The characterization of its biochemical activity and the identification of the possible targets are of great interest and will warrant further investigation.

Methyltranferases are a large family of proteins that are involved in methylation of a wide variety of substrates including DNA, RNA and proteins and the atomic targets can be carbon, oxygen, nitrogen and sulfur. However, in most cases, no specific traits in the sequence or the structure can be reliably used to predict the substrate of the modification. Indeed, motif I is present in DNA, RNA, protein and small molecule methyltransferases (Kagan and Clarke, 1994). In mitochondria, methylation has an essential role. Mitochondrial DNA, tRNA and rRNA are all targets of specific methylation events (Helm et al., 1998; Pintard et al., 2002) and specific carriers transport SAM into the mitochondria (Agrimi et al., 2004). As far as protein methylation is concerned, only two methylated subunits have been detected in complex I subunits. One of them is the bovine NDUFB3 (B12), which is methylated at conserved His residues. Interestingly, the other one is the human NDUFS2, which harbors a methylated arginine, R323. Of course, the interaction of MidA with NDUFS2 and subsequent methylation of the subunit is an attractive hypothesis that remains to be investigated. The possible functional relevance of NDUFS2 methylation is not known but it is likely that this post-translational modification in such an important core subunit alters the assembly or the stability of the whole complex. In fact mutations in Arg228, Ser413 and Pro229 of NDUFS2 have been described to be involved in CI disease (Loeffen et al., 2001). However, we should also consider the possibility that MidA has a dual function as a chaperone and as a methyltransferase. MidA is not stably bound to CI, as suggested by our results using BN-PAGE. Therefore, it is possible that the interaction with NDUFS2 occurs during the assembly process of

the complex, thus functioning as a transitory step that is required for correct CI stability.

The complex phenotypes of *Dictyostelium* cells deficient in MidA show similarities with other strains with mitochondrial disease, but there are also differences. Defects in phototaxis and thermotaxis have been described previously in other *Dictyostelium* mitochondrial dysfunctions that affect respiration, such as ethidium-bromide-mediated mtDNA depletion and the antisense inhibition of Chaperonin 60 (Cpn60) (Bokko et al., 2007; Chida et al., 2004; Kotsifas et al., 2002; Wilczynska et al., 1997). Interestingly, the phototaxis impairment in the MidA mutant can be rescued by AMPK inhibition, similarly to the other described mitochondrial mutants. However, *midA*$^{-}$ cells showed a severe defect in phagocytosis and macropinocytosis: a phenotype that is not rescued by AMPK antisense inhibition and is not even present in the other described mitochondrial mutants. This defect would, in turn, explain the AMPK-independent impairment of growth that we observe in the MidA-null mutant. The results suggest that either MidA itself or CI activity are specifically required for normal phagocytosis and pinocytosis.

We observed compensatory responses that increased the expression and level of activity of other respiratory chain complexes and the amount of mtDNA in the cells. This suggests a feedback that stimulates mitochondrial biogenesis and ATP production, but fails to correct the specific CI deficiency caused by the absence of MidA. AMPK might participate in this feedback, because it stimulates mitochondrial biogenesis and ATP production in *Dictyostelium*, as in other organisms (Bokko et al., 2007). The pattern of phenotypic outcomes in the MidA-null mutant thus results from both chronic AMPK activity and a specific failure of the associated feedbacks to correct the CI deficiency. This reveals once more the complexity of mitochondrial cytopathology and specifically in CI diseases. The observation of the role played by AMPK signaling in complex I pathology in *Dictyostelium* suggests that in humans some of the associated phenotypes might also be mediated by chronic activation of this signaling pathway. If so, treatment aimed to regulate AMPK signaling might be beneficial.

Materials and Methods

Dictyostelium growth, transformation and development

Dictyostelium AX4 cells were grown axenically in HL-5 medium or in association with *Klebsiella aerogenes* in SM plates (Sussman, 1987). Transformations were carried out by electroporation, as described previously (Pang et al., 1999). For synchronous development, axenically growing cells were washed from culture medium by centrifugation, resuspended in water or PDF buffer and deposited on nitrocellulose filters (Shaulsky and Loomis, 1993).

Human cell culture and RNA interference

HEK293T and HeLa cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown following the specifications of the repository. Two different micro RNA adapted short-hairpin RNAs (shRNAmir) cloned into pGIPZ vector (V2HLS_31857 and V2HLS_31862; Open Biosystems) were used to stably knockdown the human gene encoding MidA (*C2orf56*). Lipofectamine 2000 (Invitrogen) was used for transfection of the constructs according to the manufacturer's protocol. For selecting stable cell lines, the puromycin drug-resistance marker was used. Relative quantitative real-time PCR was carried out to estimate the knockdown levels of the human gene in a 7900HT Fast Real Time PCR system (Applied Biosystems). For this purpose, two different TaqMan assays were used. One specifically detected the levels of mRNA encoding MidA (Hs00218600; Applied Biosystems). The other was a TaqMan ribosomal RNA control designed to detect the 18S ribosomal RNA gene as endogenous control (4308329; Applied Biosystems).

Mitochondrial localization

Mitochondrial localization with Mitotracker Red (Molecular Probes) was performed as previously described (Torija et al., 2006b). Confocal analysis was performed in a Leica TCS SP5 using a PL APO 63×/1.4-0.6 objective and LAS-AF (Leica Application Suite) software.

Spectrophotometric analysis of the OXPHOS complexes and BN-PAGE

For spectrophotometric analysis, 5×10^7 growing cells were centrifuged and washed once with PBS. The pellet was resuspended in 2 ml SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl, 100 U/l heparin, pH 7.4) and then sonicated three times in ice-cold water for 10 seconds with 30 second rests in between. To eliminate cell debris, the sample was centrifuged and the supernatant was used as reaction sample for spectrometric analysis, as previously described (Tiranti et al., 1995).

BN-PAGE analysis was basically performed as previously reported (Calvaruso et al., 2008) with small changes. Briefly, 1×10^7 cells were resuspended in 3× gel buffer (750 mM aminocaproic acid, 150 mM Bis-Tris pH 7.0) and the amount of protein quantified with the Bradford assay. N-dodecyl β-D-maltoside (Sigma) was added to a ratio 20 µg per µg total protein and made up to 40 µl with 3× gel buffer. 40 µg were loaded per lane for the CI detection, whereas 10 µg were loaded for MidA detection. For western blot analysis, the gel was transferred overnight at room temperature to a 0.45 µm PVDF membrane (PALL life sciences) at 30 V with 1× Tris-Gly, 20% methanol and 0.02% SDS buffer. Then, the membrane was stripped with 2% SDS, 62.5 mM Tris-HCl, pH 6.8, for 90 minutes. The western blot was carried out with total OXPHOS Human WB Antibody Cocktail (Mitosciences) to assay CI stability. A protein standard (Invitrogene) was used to estimate the size of the complexes. Secondary antibody goat anti-mouse IgG-HRP was provided by Santa Cruz Biotechnology. For MidA dimer experiments, the second dimension was made as previously described (Calvaruso et al., 2008). Anti-GFP (SIGMA) and goat anti-rabbit IgG-HRP (Santa Cruz) antibodies were used.

Site-directed mutagenesis

Site-directed mutagenesis of G170V and G172V was performed by PCR using as template the complete MidA gene cloned in pGEMt. Two complementary oligonucleotides containing the desired mutations were used. For G170V: oligo 1, CAA ATA GTT GAA ATG GTT CCA GGT AGA GGC ACA CTA ATG; oligo 2, CAT TAG TGT GCC TCT ACC TGG AAC CAT TTC AAC TAT TTG. For double mutation G170V and G172V: oligo 3, CAA ATA GTT GAA ATG GTT CCA GTT AGA GGC ACA CTA ATG. Oligo 4, CAT TAG TGT GCC TCT AAC TGG AAC CAT TTC AAC TAT TTG. The PCR reaction was digested with *Dpn*I and transformed into *E. coli* DH5α for plasmid amplification. The constructs were fully sequenced to confirm the mutations and cloned in-frame with GFP in the vector pDV-CGFP-CTAP, kindly provided by Pauline Shaap (University of Dundee, Dundee, UK). A similar construct was used with a complete wild-type sequence of MidA.

Pull-down assays

The N-terminus of *Dictyostelium* and human genes encoding NDUFS2 were expanded by PCR and cloned in PGEX plasmid (Pharmacia-Biotech) for bacterial expression and subsequent purification by the GST system according to the manufacturer's instructions. The *Dictyostelium* NDUFS2 construct spanned amino acids 8-176 and human NDUFS2 amino acids 38-234. GST alone was used as a control. The isolated proteins were kept associated with the Sepharose beads until used in the pull-down assay. 5×10^6 *Dictyostelium* and HEK293T cells expressing *Dictyostelium* or human MidA fused to GFP were resuspended in 500 µl STE+T buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA pH 8, 150 mM NaCl, 5 mM DTT, 1% Triton X-100, 1× protease inhibitor cocktail) and shaken for 30 minutes at 4°C. After centrifugation, the supernatant was incubated with 30 µl of previously isolated proteins bound to the Sepharose beads for 1 hour at 4°C. Beads were washed three times with STE+T and finally resuspended in 30 µl protein loading buffer. The sample was split in two SDS-PAGE gels. One was stained with Coomassie Blue as a control and the other was transferred to PVDF for western blotting using anti-GFP antibody.

mtDNA and mtRNA quantification

For mtDNA quantification *Dictyostelium* cells were grown in HL-5 medium to log phase. Genomic DNA from 6×10^6 cells was extracted with 300 µl Quick Extract DNA Extraction Solution 1.0 (Epicentre). 1:50 dilution was used as a template in the PCR reaction carried out in 7900 HT Fast Real-Time PCR System, using Power Sybrgreen PCR Master Mix 2 with 300 nM oligonucleotides in a final volume of 10 µl. Results were acquired with SDS 2.3 software by Applied Biosystems and handled with Excel software by Microsoft. Two pairs of oligonucleotides were used: One for detection of mtDNA (DDB_G0294054), oligo1, AAC AAT CAT GTG GCT TTA GTA CGT AAA; oligo2, TCG GCC CTG CAT TTC GT and another for normalization with a nuclear gene (DDB_G0277273). Oligo 1, CCG TTG CCC TAA CTT ACT TCC A; oligo 2, GCC GCC ATT GAT GAA ACT ATT C. For mtRNA quantification, RNA from 1×10^7 cells growing to log phase in HL-5 medium was isolated with Tri-Reagent (Sigma) and adjusted to 2 µg/µl final concentration. DNA contamination was removed by adding 50 U DNase (New England Biolabs) to 10 µg RNA in a final volume of 10 µl. The reaction was incubated for 30 minutes at 37° C following an incubation at 70°C for 5 minutes to destroy DNase activity. The RNA was then adjusted with DEPC water to 100 ng/µl final concentration. 250 ng (2.5 µl) of this RNA was used as a template for RT-PCR with high capacity cDNA reverse transcription kit (Applied Biosystems) in a final volume of 20 µl. The cDNAs served as template in the PCR reaction carried out as described above. Eight genes

representative of the previously described eight major polycistronic transcripts (Barth et al., 2001) were studied for each sample, normalized to the nuclear gene DDB_G0277273 and referred to the wild-type levels. The oligonucleotides used are listed in supplementary material Table S2.

Phototaxis and thermotaxis assays

Qualitative phototaxis tests were performed as described (Darcy et al., 1994) by transferring a toothpick scraping of amoebae from a colony growing on a *K. aerogenes* lawn to the center of charcoal agar plates (5% activated charcoal, 1% agar). Phototaxis was scored after a 48 hour incubation at 21°C with a lateral light source. Quantitative phototaxis tests involved the harvesting of amoebae from mass plates, thoroughly washing them free of bacteria, suspending them in saline at the appropriate dilutions and inoculating 20 µl onto a 1 cm^2 area in the center of each charcoal agar plate. The resulting cell densities ranged from about 1.5×10^6 to 3.7×10^7 cells/cm^2. The phototaxis was again scored after a 48 hour incubation at 21°C with a lateral light source. Quantitative thermotaxis used washed amoebae prepared as for quantitative phototaxis and plated at a density of 3×10^6 amoebae/cm^2. A 20 µl aliquot of cells at this dilution was plated on a 1 cm^2 area in the center of water agar plates (1% agar) and incubated for 72 hours in darkness on a heat bar producing a 0.2°C/cm gradient at the agar surface. The arbitrary temperature units correspond to the temperatures 14°C at T1 and increasing in 2°C increments to 28°C at T8, as measured at the center of plates in separate calibration experiments. Slug trails were transferred to PVC discs, stained with Coomassie blue and digitized. The orientation of the slug migration was analysed using directional statistics (Fisher, 1981).

Phagocytosis and pinocytosis assays

All *Dictyostelium* strains (wild type AX2, midA null mutant and the midA rescued strain) were grown in HL-5 medium with no antibiotics to exponential phase before use in the pinocytosis and phagocytosis experiments. Bacterial uptake by *Dictyostelium* strains was determined by using as prey an *E. coli* strain expressing fluorescent protein DsRed (Maselli et al., 2002) as previously described (Bokko et al., 2007).

Pinocytosis assays (Klein and Satre, 1986) were performed by measuring the uptake of medium containing a fluorescent indicator, fluorescein isothiocyanate (FITC)-dextran (Sigma, average mol. mass 70 kDa), as previously described (Bokko et al., 2007).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/10/1674/DC1

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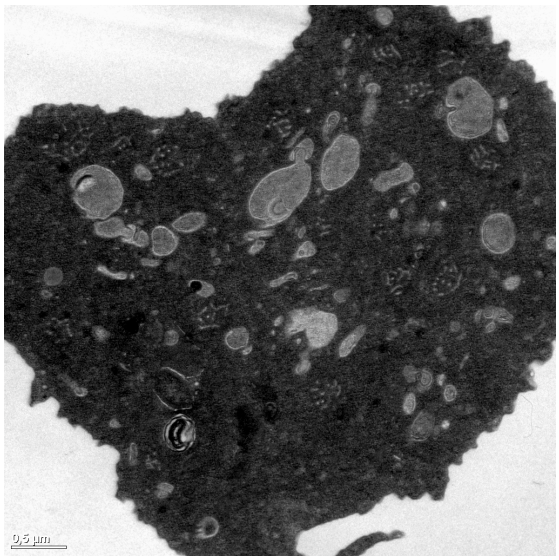
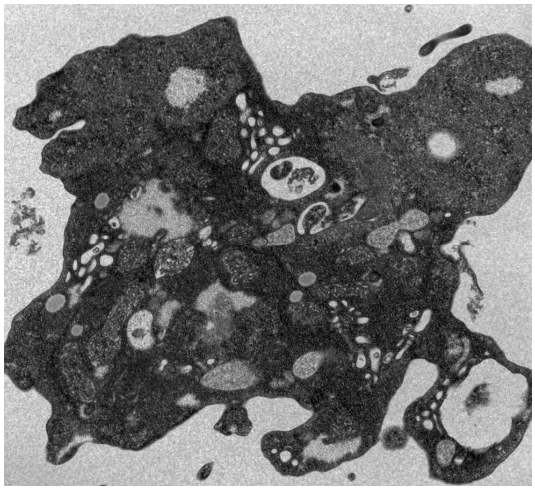


ANEXO II: MATERIAL SUPLEMENTARIO



MATERIAL SUPLEMENTARIO CAPÍTULO 1

E08-01-0075 Escalante

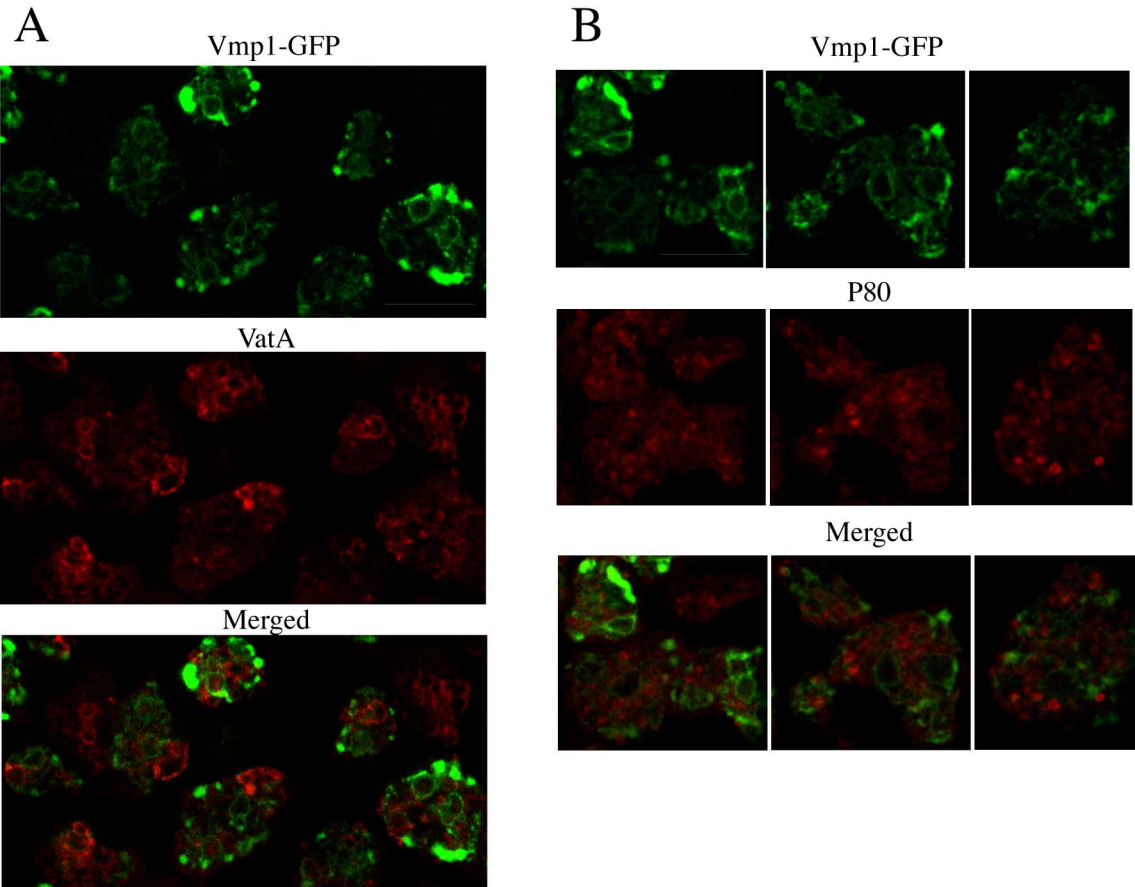
Supplementary Movie 1. Cell lysis in water. Mutant cells were taken from SM plates and deposited in water. After 15 minutes of incubation, cells were recorded for 14 seconds in a phase contrast Nikon Eclipse TS100 microscope.

Suplementary figure 1. Vmp1 protein is not located in endosomes and contractile vacuoles. The complemented strain expressing the fusion Vmp1-GFP protein was treated for immunodetection of the CV/endosomal marker VatA (A) and the endosomal marker p80 (B). The samples were analyzed by confocal microscopy and no colocalization was detected. Scale bars: 10 μm.

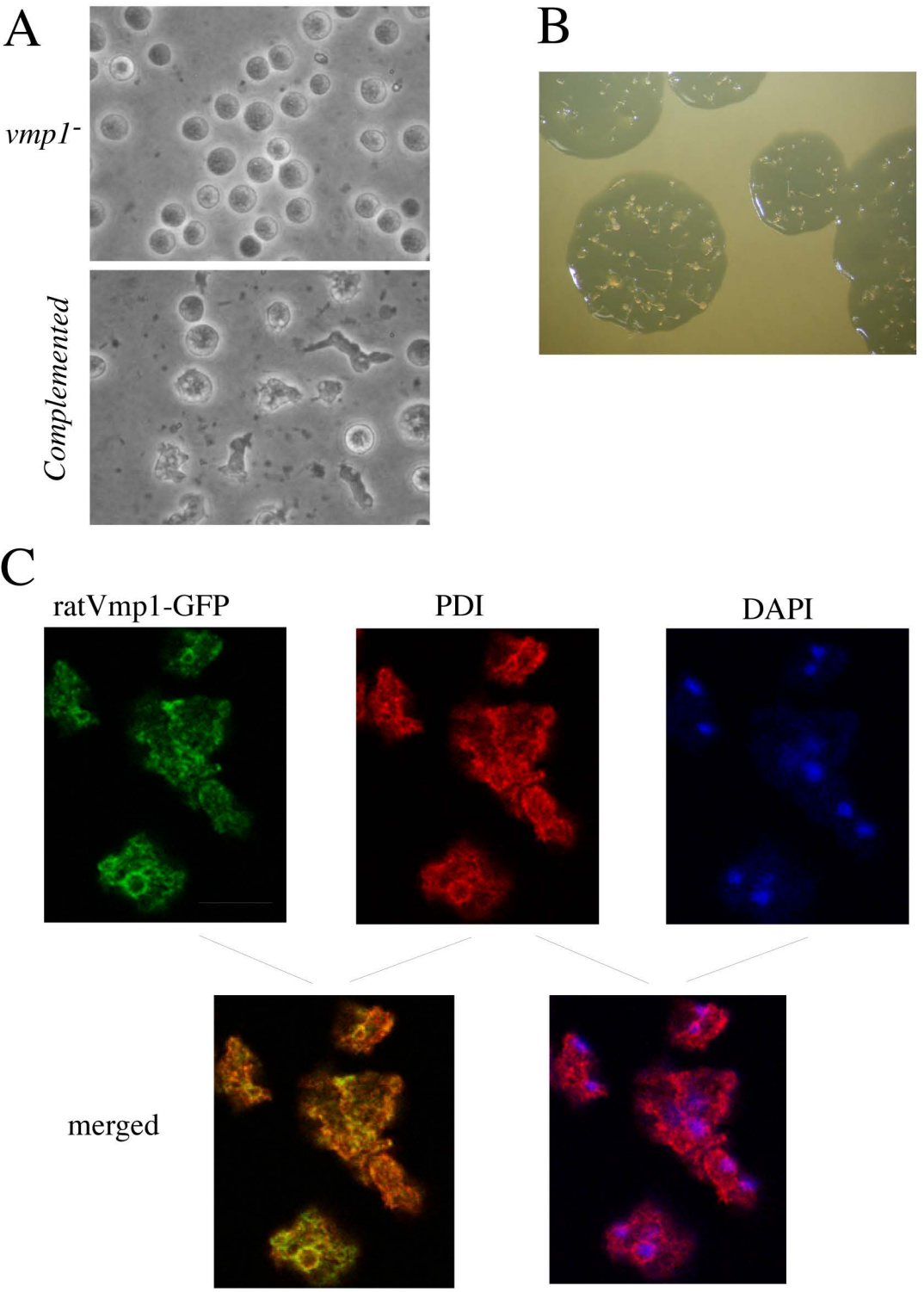
Supplementary Figure 2. Mammalian and *Dictyostelium* Vmp1 are functionally equivalent. *vmp1*$^-$ cells were transformed with an expression vector containing the rat Vmp1 fused with GFP. The transformant strains showed wild type phenotype. (A) Mutant and complemented strain were exposed to water during 1 hour. Complemented cells showed irregular shape and vacuoles. (B) Complementation of development in SM plates. Mature fruiting bodies can be distinguished in the mutant. (C) The complemented strain was used for confocal analysis. Antibodies against the ER marker PDI (red) colocalized with Vmp1-GFP (green). Nuclear DAPI staining showed in some cases clear green fluorescence in the nuclear envelope region. Bar: 10 μm.

Supplementary figure 3. Single crossover strategy. The expression plasmid containing *Dictyostelium* Vmp1 fused to GFP was transformed in the *vmp1*$^-$ mutant strain. A low proportion of the transformants showed single crossover events as illustrated in the scheme. Amplification from oligonucleotides 1 and 2 was used to identify the single crossover. The amplified PCR fragment was sequenced for confirmation. Transformants containing random multicopy insertions gave no PCR amplification. The level of GFP fluorescence in the single crossover strain was lower than that obtained in the multicopy-insertion strains. Nevertheless the GFP fluorescence was localized in the ER.

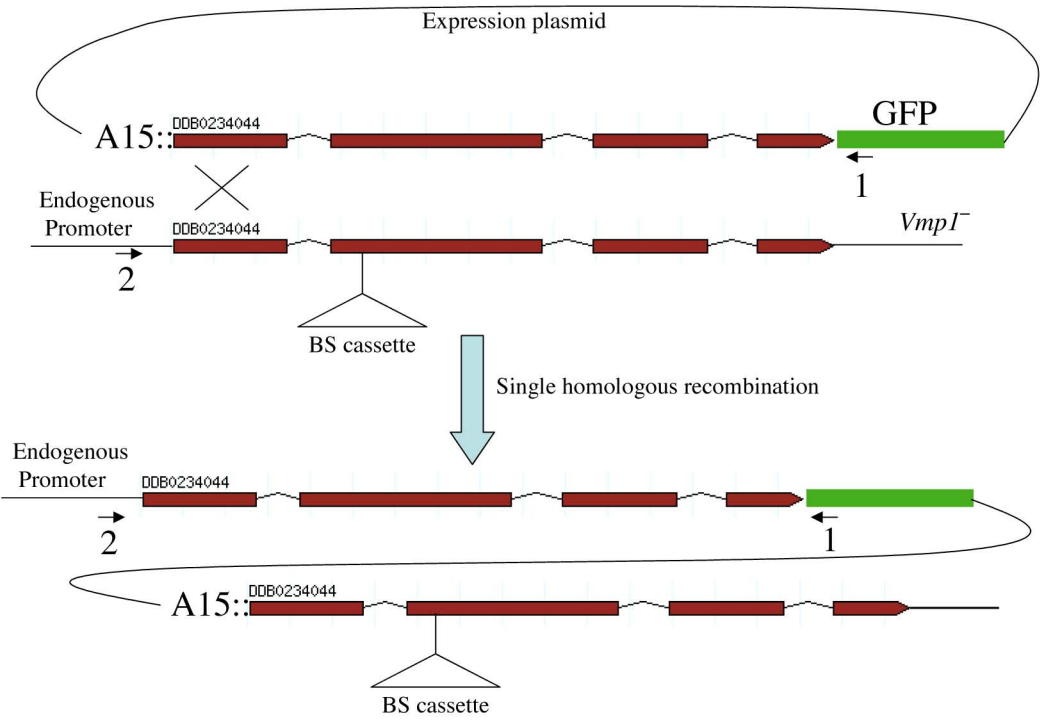
Sup. FIGURE 1



SUP. FIGURE 2



Sup. FIGURE 3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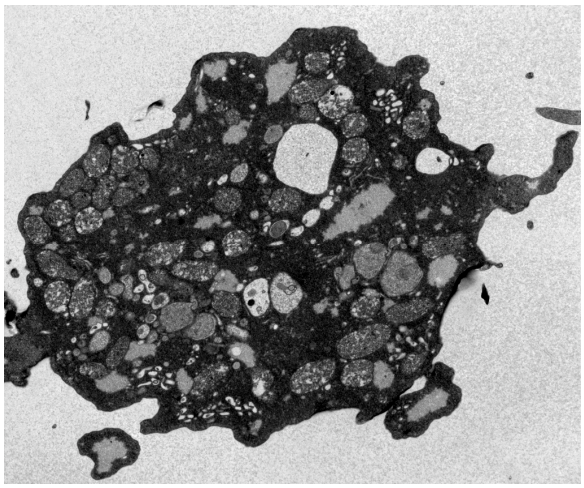
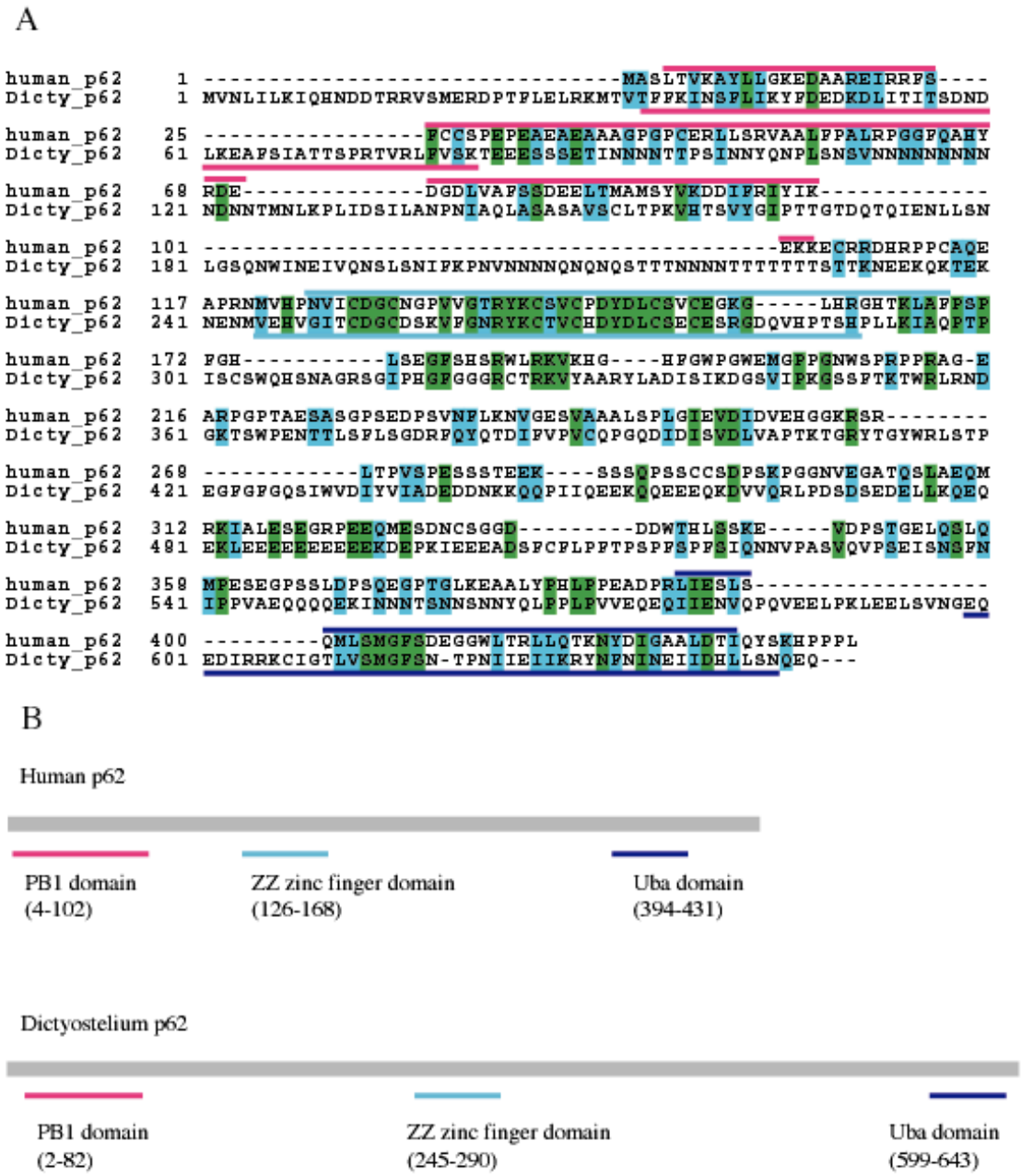
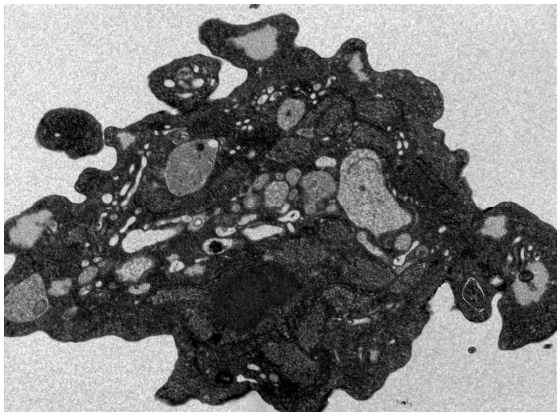
DDB0234044
GFP
1
Endogenous
Promoter
DDB0234044
Vmp1⁻
2
BS cassette
Single homologous recombination
Endogenous
Promoter
DDB0234044
2
1
A15::
DDB0234044
BS cassette



MATERIAL SUPLEMENTARIO CAPÍTULO 3



Supplementary figure 1. Sequence analysis of a putative *Dictyostelium* p62 gene. (A) Sequence alignment of human and *Dictyostelium* p62 predicted proteins using the ClustalW program. Green corresponds to identical residues and blue to similar residues. The position of the functional domains was marked by a colored line above the sequence (for human p62) and below the sequence for Dictyostelium p62. (B) Scheme of the functional domains in human and *Dictyostelium* proteins. Functional domains are displayed by different colors. The same code of colors was used to position the domains over the sequence in A.



MATERIAL SUPLEMENTARIO CAPÍTULO 4

Sup. Table 1. Proteins involved in selective autophagy (Cvt pahway and Mitophagy)*

	Function/features	Dictyostelium	Human	*S. cerevisiae*	E-value Dd-Hu	E-value Dd-Sc
Atg11	Adapter protein.(Cvt)	---------	---------	Atg11(YPR049C)		
Atg19	Receptor protein. (Cvt)	---------	---------	Atg19(YOL082W)		
Atg20	Phosphoinositide binding Phox-domain protein.(Cvt)	---------	---------	Atg20(YDL113C)		
Atg21	WD repeat domain phosphoinositide-interacting protein (Cvt)	Atg18(DDB_G0285375) Wdr45l(DDB_G0282581)	WIPI-2(G.ID: 26100) Other homologues (WIPI-1; WDR45L/WIPI-3)	Atg21(YPL100W)	2e-51 4e-32	4e-12 2e-10
Atg24	Phosphoinositide binding Phox-domain protein. (Cvt)	Vps5(DDB_G0272989)	SNX30(G.ID401548) Sorting nexin family	Atg24/SNX4 (YJL036W)	1e-12	2e-4
Atg32	Mitochondrial-anchored transmembrane receptor. (Mit)	---------	----------	Atg32(YIL146C)		
Atg33	Mitochondrial mitophagy-specific protein. (Mit)	---------	----------	Atg33(YLR356W)		
Vac8	Vacuolar membrane protein that interacts with Atg13. Armadillo repeat protein. (Cvt)	---------**	----------**	Vac8(YEL013W)		
Vps38	Part of the PtdIns3K (Vps34)- complex (Cvt)	----------	----------	Vps38(YLR360W)		

*Atg genes not included: Atg25,Atg28 and Atg30 are required for pexophagy in other yeast species but are not conserved in *S. cerevisiae*. Atg26 is not involved in autophagy in *S. cerevisiae*.

** Low homology restricted to the armadillo-repeat domain can be detected in multiple proteins.